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Two New Approaches to the Identification of Candida Albicans‡

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The identification of *Candida albicans* continues to be a serious problem in many clinical laboratories. Failure to differentiate the pathogenic *Candida albicans* from the other non-pathogenic species can result in incorrect diagnoses and unnecessary therapy. Even though carbohydrate fermentation studies have been shown to be reliable, these reactions are often found impractical for routine use. Animal inoculations and serological studies can also be employed, but again these procedures are laborious and time-consuming.[3]

Modifications of the slide method for the culture of pathogenic fungi, [1, 5, 6, 7] which was introduced in 1930 by Henrici,[4] have proved to be practical, economical, and useful in the study of those fungi whose identification depends entirely upon morphological characteristics such as size, location, and arrangement of spores. Utilization of this technique provides a reliable means for the identification of *Candida albicans* since it can be distinguished from the other species of the genus chiefly by its characteristic production of chlamydospores on a nutrient-poor medium such as corn meal agar.

The present paper presents a coverslip adaptation of the slide culture technique which is relatively easy to accomplish and which has proved extremely dependable in our laboratory. Since only recently have new media recommended for direct isolation and identification of *Candida albicans* become commercially available, we chose to compare results obtained by our modification with those obtained by using one of these newer media, Pagano-Levin culture medium.*

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* The Pagano-Levin culture medium was supplied through the courtesy of the E. R. Squibb Company.

Materials and Methods

Modified Slide Culture Technique: The materials necessary for the cultures are shown in Fig. 1. As can be observed, the equipment is simple and no special apparatus is required.

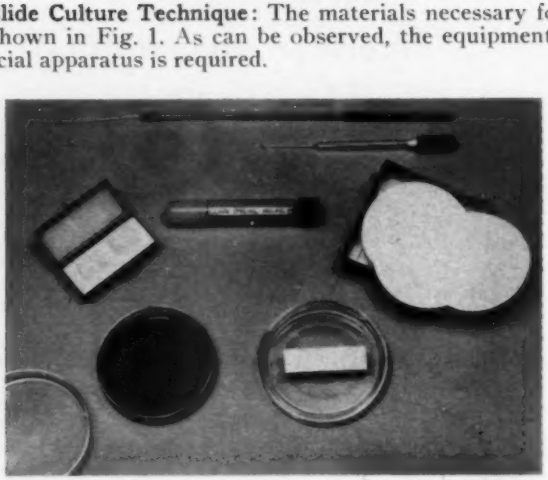

Figure 1. Materials necessary for the Modified Slide Culture Technique.

Previous studies in our laboratory have shown that freshly made corn meal agar yields better results than the commercially available media provided for stimulating chlamydospore production. We have found also that yellow corn meal is superior to white meals. The specific procedure by Conant et al.,[2] is recommended for the preparation of the agar. It is best stored in screw-cap tubes at refrigerator temperature. Just prior to use, the agar is melted by placing the tube in a beaker of boiling water. It is cooled to approximately 45°C. for inoculation.

Colonies suspected of being Candida developing on B.B.L. Sabouraud Dextrose Agar from clinical material are sub-cultured to plates of Difco Blood Agar Base and incubated at 37°C. for 24 hours.

A sterile Petri dish is labeled by sticking a strip of white tape on the bottom of the plate and numbering it in ink with the corresponding culture number. A red or black china-marking pencil can be used directly on the plate to label the cultures, but these markings often smudge and rub off onto the microscope stage during the examination. Since coverslips require little space, four cultures can be made in one Petri dish.

Two to three drops of sterile melted corn meal agar are dropped from a short-tipped, sterile Pasteur pipette directly onto a quadrant of the Petri dish. Using a wire loop, a small amount of inoculum from the sub-culture is deposited onto the medium. Immediately, a clean coverslip is placed over the medium in such a way as to assure the exclusion of air. The Petri dish has remained open during this procedure. The cover is replaced and the cultures are left undisturbed until the media have hardened.

Next two sheets of filter paper. Whatman No. 1, 9.0 cm. in diameter, are placed in the Petri dish cover and moistened with tap water. The bottom of the Petri dish, containing the coverslip cultures, is then inverted and placed over the cover. The Petri dish set-up remains in this inverted position during incubation at room temperature. Four completed cultures have the appearance shown in Fig. 2.

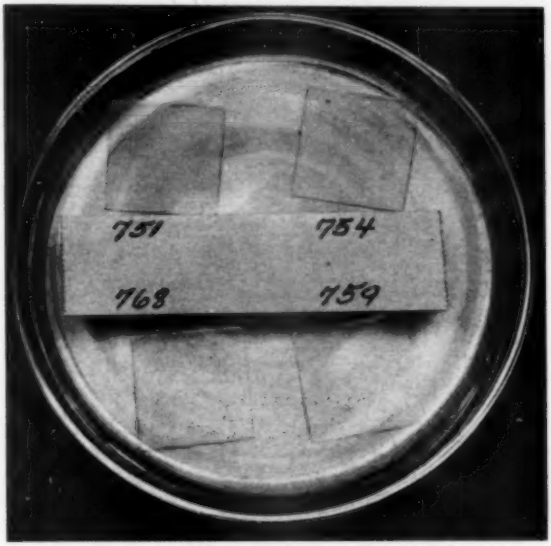

Figure 2. A Petri dish set-up containing four completed cultures.

After 24 hours, the Petri dish is uncovered and the bottom containing the cultures is placed under a microscope to be examined for chlamydospore production. It has been found more convenient to remove the mechanical stage of the microscope for this examination. The condenser is lowered to reduce the light. Since the layer of agar between the plate and the coverslip is thin, optical conditions are good. Since many of the chlamydospores are approximately in the same plane, their detection is easy. A preliminary examination is made using the low power objective, but the high power objective is necessary to see the detailed structure of the chlamydospores. By introducing a small amount of lactophenol cotton blue around the coverslip, the chlamydospores become more distinct by absorbing the stain.

The majority of chlamydospores are seen within 24 hours; occasionally they are noted for the first time after an incubation of 48 hours. For this reason no culture is reported negative until a 48 hour reading is made. Fig. 3a shows a microscopic view of chlamydospore formation; Fig. 3b shows a microscopic view of a species of *Candida* which failed to produce chlamydospores.

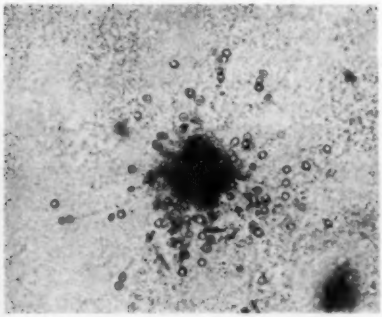

Figure 3a. A microscopic view of chlamydospore formation, Lactophenol Cotton Blue Stain, X180

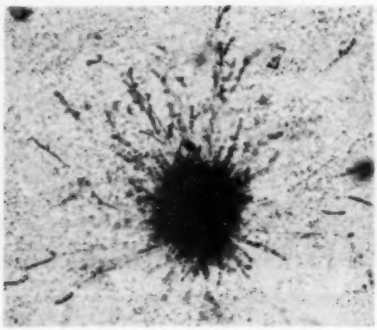

Figure 3b. A microscopic view of *Candida* which failed to produce chlamydospores, Lactophenol Cotton Blue Stain, X180.

Pagano-Levin Medium Technique: As shown in Fig. 4, the medium is supplied in 2-dram vials ready for immediate use. The directions for use as well as two sterile cotton-tipped applicators are also supplied.

By using the sterile swab the clinical material from a suspected *Candida* infection is streaked directly onto the agar. The cap of the vial is replaced, but is not closed tightly. The culture is incubated for 2 to 3 days at room temperature. At this time the color intensity of the colonies provides a rapid and convenient means of distinguishing *Candida albicans* from other species. *Candida albicans* produces creamy white or faintly pink colonies which are smooth and elevated. Other *Candida* species and other yeasts appear as dark pink to red colonies. *Candida krusei* may appear as chalky white colonies, but these can usually be differentiated by their flat, dry wrinkled appearance. Occasionally bacterial colonies may appear which are generally red in color.

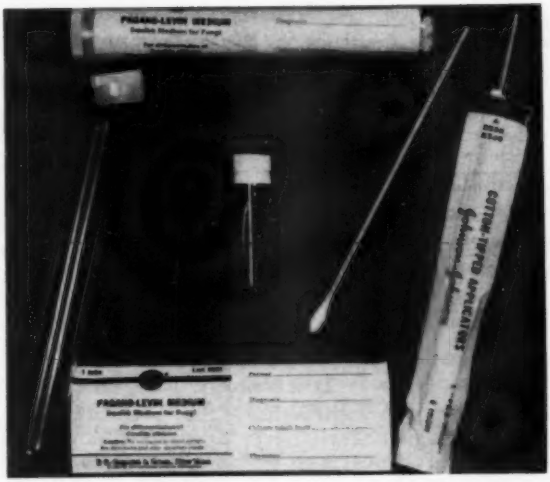

Figure 4. The Pagano-Levin Culture Medium kit.

Results

With the cooperation of the Department of Obstetrics and Gynecology, a total of 1500 cultures were taken from the vagina and breasts of 375 selected obstetrical patients. Each vaginal culture and each breast culture were planted directly on vials of Pagano-Levin medium and tubes of Sabouraud's dextrose agar. If colonies characteristic of *Candida* grew on the Sabouraud's dextrose agar, our slide culture modification was employed for chlamydospore formation.

Since different species of *Candida* will grow readily on Sabouraud's dextrose agar, it was deemed necessary to evaluate Pagano-Levin medium in this regard. Results of this comparison are show in Table I. Of the cultures positive for all species of *Candida*, 96% grew on Sabouraud's dextrose agar while 92% grew on the Pagano-Levin medium.

Final identification of *Candida albicans* was based upon the observation of chlamydospore formation utilizing the slide culture modification and upon the color appraisal of the colonies growing on the Pagano-Levin medium. A comparison of these positive findings is shown also in Table I. One hundred percent of the positive cultures of *Candida albicans* were identified with the modified slide method while the Pagano-Levin medium revealed 93%. Except for *Candida albicans*, no attempt was made to identify other species of *Candida*.

It is of interest to note that 33.9% of all vaginal cultures and 8.5% of all breast cultures were positive for all species of *Candida* on either Pagano-Levin medium or Sabouraud's dextrose and corn meal agar while 10.7% of all vaginal cultures and 0.8% of all breast cultures were positive for *Candida albicans*.

TABLE I

Results of Positive Cultures Comparing the Pagano-Levin Medium with Sabouraud's and Corn Meal Agar

	Total No. Pos. Cults. on Either P-L Medium or Sab. and Corn Meal Agar	Pos. Cults. on Sab. and Corn Meal Agar		Pos. Cults. on Pagano-Levin Medium	
		No.	%	No.	%
Pos. vaginal cults. for **Candida** (all species)....	127	124	98	120	94
Pos. vaginal cults. for **Candida albicans**.....	40	40	100	38	95
Pos. breast cults. for **Candida** (all species).....	32	30	94	27	84
Pos. breast cults. for **Candida albicans**.....	3	3	100	2	67
Total no. pos. cults. for **Candida** (all species)..	159	154	96	147	92
Total no. pos. cults. for **Candida albicans**....	43	43	100	40	93

Discussion

The results of this experiment indicate that both the coverslip culture technique and the use of Pagano-Levin culture medium provide reliable means of isolating and identifying *Candida albicans*. The coverslip method as presented here, appears somewhat superior to that of the direct isolation method.

The modifications in our coverslip technique were directed toward obtaining results as quickly as possible without sacrificing accuracy. One time-consuming factor is the necessary sub-culturing of *Candida* to remove dextrose which would prevent chlamydospore formation.[3] Even though it has been recommended that a second sub-culture be incubated for 48 hours before inoculation onto chlamydospore media, we found one sub-culture to be satisfactory, thus saving 24 hours.

One difficulty often arising from the use of microcultures is the problem of contamination. We found that coverslips taken directly from their original container were suitable and that no contamination was encountered by dropping the coverslips by hand. In like manner, non-sterile filter paper moistened with tap water to provide a humid atmosphere in order to reduce media dehydration, resulted in no contamination. All equipment other than the coverslips and filter paper were sterile and aseptic technique was employed.

Another major advantage that this modification offers is that of convenience of examination of the microcultures. The procedure as given allows for a direct and simple inspection for chlamydospores at both 24 and 48 hours without disturbing or contaminating the cultures.

With the advent of newer media for the direct isolation and identification of *Candida albicans*, a method has been made available which eliminates the necessity of sub-culturing and allows for a direct visual identification of this fungus.

The Pagano-Levin culture medium was developed by Pagano, et al.,[8] in 1957. The differentiation of *Candida albicans* from other *Candida* species as well as other yeasts is accomplished on this medium due to the presence of a biological indicator, 2,3,5-triphenyltetrazolium chloride. The different yeasts have varying capacities to reduce a given concentration of this compound resulting in different color intensities. The

presence of the broad spectrum antibiotic, neomycin, suppresses the growth of bacteria.[8]

The Pagano-Levin culture medium, as our results show, compares favorably, but not absolutely with the coverslip culture procedure. However, the number of positive cultures obtained by the former method are significantly consistent with the number obtained by the latter as indicated in Table I. The apparent discrepancy in the percentage of positive breast cultures for *Candida albicans* is due to the fact that only three positive cultures were obtained.

This new media's chief value seems to lie in the fact that it provides a rapid and simple means of identifying *Candida albicans*. The inexperienced observer as well as the medical technologist without facilities for alternate methods, can take advantage of this method.

Summary and Conclusions

1. A coverslip modification of the slide culture technique and a new medium, Pagano-Levin culture medium, were compared in the identification of *Candida albicans*. One hundred percent of the positive cultures of *Candida albicans* were identified with the modified slide method while the Pagano-Levin medium revealed 93%.

2. The coverslip modification of the slide culture technique as presented in this paper provides a rapid and convenient means for the identification of *Candida albicans*. This modification requires limited materials and reduces contamination to a minimum.

3. The use of Pagano-Levin culture medium provides a reliable and simple means of isolating and identifying *Candida albicans* eliminating the necessity for sub-culturing.

Acknowledgment

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Preparation of Cerebral Tissue to Evaluate Reactions to Metal Implantation*

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Introduction

The selection of metal electrodes for implantation in the brain of an experimental animal for the purpose of recording or stimulation may be limited by the tissue reaction to the metals. The tissue must be carefully perfused, and specific appropriate stains must be selected to study the cellular response at the electrode sites. The metals showing the least damage to the surrounding tissue are the materials of choice for future electrodes.

A thorough pathological study of tissue reaction to relatively large (600 micron diameter) coated, implanted electrodes was reported by Collias and Manuelidis.[1] They used bromformol solution for perfusion and employed seven different histological stains. Marshall[9] described an alcohol-formalin fixation method with special advantages for brain tissue. Fox and Eichman,[2] in electrode studies, applied a photographic technique to thick sections of brain. The stages in pathological reactions of neuroglia were discussed and illustrated by Rand and Courville[10] who also evaluated the results of several stains.

A description of perfusion technique and special stains for central nervous system pathology is presented.

Method

The metals used in this study were implanted surgically in the brains of healthy cats.[11] Three metal rods, gold, platinum, and silver, were placed in the left hemisphere and three others, stainless steel, tantalum, and tungsten, in the right hemisphere. The diameter of each rod was 125 microns. Sixteen cats were utilized in the study. Two animals were sacrificed after each of the following post operative recovery periods: 1 day, 3 days, 7 days, 15 days, 1 month, 2 months, 4 months, and 6 months.

Perfusion. Perfusion was performed after the animal had been injected with an excessive amount of sodium pentobarbital and was completed before vascular clotting occurred in the post mortem state. A rapid and more effective fixation of tissue was accomplished by perfusion of the blood vessels with physiological saline followed by a fixative.

One liter bottle of 0.9% saline and one of 10% formalin were connected by polyethylene tubing to a cannula. The operative procedure of Jones[5] for exposing the heart and aorta was followed. Cannulation was performed by way of the left ventricle into the aorta, where the cannula was firmly secured. Extravasation was effected by an incision in the right atrium, and the aorta was clamped at the diaphragm to limit perfusion to the anterior structures. The vascular system was washed out with physiological saline for approximately 10 seconds, followed by two liters of 10% formalin.

* Read before the 29th Annual Convention, Seattle, Washington, June 1961.

Fixation. The head was removed, placed in 10% formalin and in 24 hours the brain was removed from the skull. The metal rods were carefully removed at this time with fine forceps, and three approximately equal horizontal slices were taken through each hemisphere to encompass the lesions formed by the 12 mm electrodes; this produced cross sections of the metal implant sites. These slices were placed in 20% alcoholic formalin* and kept there 24 to 48 hours,[9] then cut on a freezing microtome without washing. For metallic stains, the remaining tissue was later transferred to formalin-ammonium-bromide solution for 24 hours at 37° C.

Sectioning. Frozen sections are required to (1) study lipid substances which are lost in paraffin embedding, (2) preserve cellular elements with less shrinkage and (3) insure metallic impregnation of neuroglia, the connective tissue of the nervous system. An additional advantage is the economy of time as freshly-cut tissue may be passed through staining solutions at once and mounted on slides, eliminating the dehydration and embedding steps required for paraffin sections. A table model Sartorius freezing microtome was used. The sections were always cut at a thickness of 20 microns so that uniform comparisons could be made.

The technique of cutting acceptable frozen sections improves with experience. Excessive freezing produces ice crystals which shatter the tissue and mar the knife edge, while insufficient freezing produces thick or uneven sections. The surface of the block should be dry, but not iced, and the knife edge should be blotted dry before each section is cut. A camel's hair brush may be dipped in water and used in a gentle sweeping motion to push each section from the knife edge and transfer it to a petri dish of distilled water where it unrolls and flattens out. Bubbles may be forced from beneath the section with the tip of the brush. The sections are mounted on albumin-coated slides and dried on a warming plate for 15 to 30 minutes at 54° C.

Staining. When tissue reactions are studied, it is advisable to use a combination of stains to better differentiate each group of cells or structures. Neural tissue, especially, has many microscopic fibers and elements which necessitate special technique for demonstration. The following stains were selected to study cerebral metal implant lesions using fresh frozen sections:

Hematoxylin and Eosin was used as a general screening stain for primary examination of tissue. The basic action of hematoxylin provides a strong nuclear stain, and the acidophilic quantities of eosin result in a valuable cytoplasmic stain.

Luxol Fast Blue-Cresyl Echt Violet, a myelin sheath stain, produces fine detail of nerve fibers with the blue pigment of copper phthalocyanine dye[6] and a well defined violet conterstain of Nissl (chrome) substance. This stain demonstrates myelin degeneration as well as chromatolysis of neurons.

Oil Red O stains fatty substances, including the products of myelin degeneration, which are engulfed by gitter, or compound granular, cells. Gitter cells appear as bright red oval or round cells on a lighter red background and are easy to distinguish because of the red granules (ingested lipids) in the cytoplasm.

* (20 ml of absolute alcohol in 80 ml of 10% formalin.)

Cajal's Gold Sublimate stain is a metallic impregnation for astrocytes, which are supportive cells in the network surrounding larger nerve cells. The astrocytes, a type of neuroglia, may show proliferative and hypertrophic changes in some lesions and are visible only with selective methods. In this stain they are dark, star-like cells with many processes or fibers on a light red or purple background.

Hortega's Silver Carbonate stain (Scharenberg modification[12]), also a metallic stain, is specific for the other two types of neuroglia, the oligodendroglia and microglia. These small cells appear black on a gray background. The microglia are usually small, oblong cells with spiked, branched processes. The colorless, round nucleus of the oligodendrocyte appears to occupy most of the cell with only a small periphery of black staining cytoplasm. After injury, both cell types may change shape, become swollen, or exhibit other pathological changes.

Hematoxylin-Eosin and Luxol Fast Blue stains may be used on mounted sections, but the metallic stains, Cajal's and Hortega's, must permeate both sides of the tissue, so that each section was passed through the solutions on a glass needle or small artist's brush. Oil Red O stain also produces better results on sections stained before mounting.

Care should be taken to flatten sections in solutions and avoid folds or curled edges. Well-fixed tissue, when properly cut on the freezing microtome, is relatively easy to handle. Complete directions for preparing solutions and for staining techniques are presented in the Appendix of a recent report.[4]

Results

The fixation technique described above resulted in optimum penetration of cerebral tissue without excessive hardening. The tissue so fixed had a white opalescent appearance with the white and gray matter clearly outlined; it was firm, but not brittle, and hard enough to section well. Varying tissue reactions to the implanted metals were revealed with the five stains used.

Hematoxylin and Eosin stain demonstrated hemorrhage, necrosis, edema, and compression of fibers in the white matter caused by the metal rods. The connective tissue in the capsule surrounding the rod, which was a stage of the healing process, was well demonstrated in some cases.

Luxol Fast Blue stain was beneficial in showing myelin and neuron degeneration, which was minimal in this study. The lesions were small because the metal rods used were only about 125 microns in diameter (Figure 1).

Oil Red O showed occasional fat droplets within the tissue and adjacent to blood vessels. Animals kept from three days to one month after implantation showed fatty products of myelin degeneration appearing as round, bright red globules within the gitter cells. The latter are usually found near the lumen of the metal site (Figure 2).

Cajal's stain gave the best indication of tissue reaction to metal and was the most sensitive stain in detecting pathological changes (Figure 3). The chief characteristics of astrocytes were clearly illustrated, i.e. the many long fibers extending from the cell bodies and the "perivascular feet" on the walls of the blood vessels. These dark star-shaped

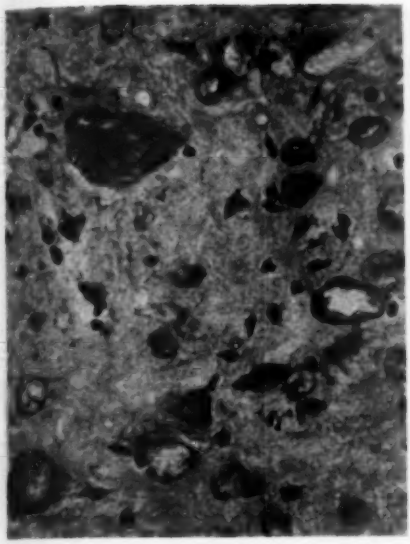

Figure 1. Neurons in brain of cat 1 day after metal implantation. Luxol Fast Blue Stain. X 460

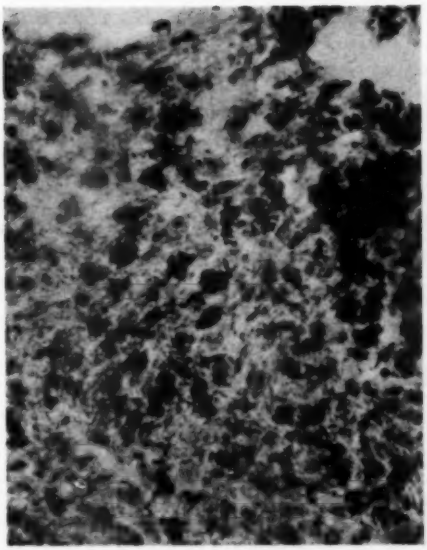

Figure 2. Gitter cells showing coarse granules of ingested lipids in area of silver implant after 7 days. Oil Red O. X 460

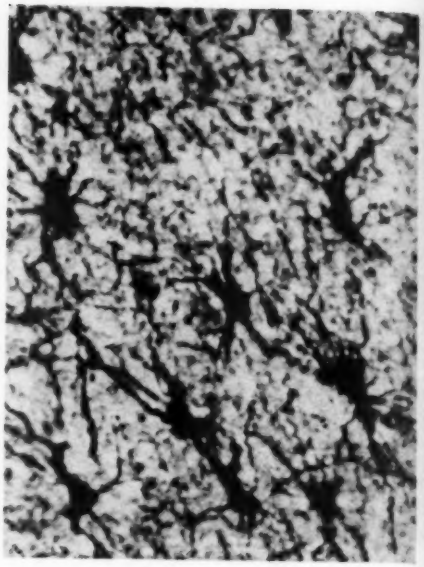

Figure 3. Cerebral tissue of cat showing astrocytes and fibrous network. Cajal's Stain. X 600

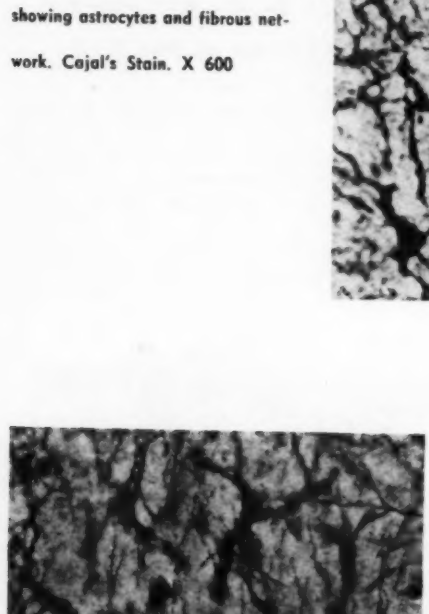

Figure 4. Microglia demonstrated as irregular or bean-shaped cells with branching processes. Scharenberg modification-Hortega's Silver Stain. X 650

cells were concentrated about the lesions and in the white matter, while neurons and most other cells were unstained.

With the modified Hortega's stain, the neuroglial cells were well defined. The small microglia were found near the lumen of the metal site and were scattered also throughout the gray matter (Figure 4). Oligodendroglia were more numerous, normally occurring in rows in the white matter, and were prominent in the periphery of the lesions.

The results of this tissue study indicated that silver implants caused the most damage to brain tissue. Tungsten was next in degree of reaction, while tantalum and platinum were about equal in producing a mild reaction. Stainless steel and gold were the least harmful of the metals used, and the changes they produced stemmed mainly from mechanical damage by insertion of the rods.

Discussion

Formalin with a weak alcoholic content was an excellent preliminary step for Hematoxylin-Eosin, Luxol Fast Blue, and Oil Red O stains, while bromformol treatment further intensified the metallic stains. The rapid and thorough penetration of the tissue slices with alcohol-formalin justifies this fixation as the preferred method.

Optimum color contrast was obtained with the five different stains as opposed to the use of any one or two of these separately. The techniques selected for staining were recent modifications of accepted methods and not only had to be specific for neural tissue but economical in time as well.

The standard Hematoxylin-Eosin technique is difficult to shorten less than 50 minutes, unless some of the steps are eliminated as previously demonstrated.[8] Luxol Fast Blue stain requires overnight incubation, but the process of differentiating and counterstaining which follows can be completed in 45 to 50 minutes. The isopropanol method for the Oil Red O staining of fats suggested by Lillie[7, 8] was effective and rapid once the staining solution was filtered; this staining procedure requires only about 20 to 25 minutes. The absence of ethyl alcohol in the procedure helps to preserve lipids in tissue and lessens precipitation of the stain. In Cajal's technique, which requires only a few solutions, the four hour incubation period can be utilized for other work. Hortega's silver impregnation, as revised by Scharenberg, requires only one and one-half hours, which is a definite saving of time in comparison to most older methods.

The time required for processing individual frozen sections with a glass needle through a number of staining solutions and the extreme care which must be exercised not to tear or wrinkle the sections are disadvantages of these five staining techniques.

The oil soluble dyes for fats, such as Oil Red O, precipitate rapidly and are stable only a few hours; in addition, the coverslips on slides must be sealed to prevent precipitation of this dye into the glycerol-gelatin mounting medium. Acid clean glassware is necessary for the metallic stains and experienced judgment is required in the timing of the modified Hortega's silver stain.

The time consumed is secondary to the effectiveness of the techniques used. Nevertheless, in the method described, fixation of neural tissues may

be shortened to three days, and sectioning and staining may be completed in one and one-half weeks. Thus a pair of animals could be processed for pathological study by one person in a period of two weeks.

Summary

Good fixation for animal cerebral tissue implanted with small diameter metal rods was obtained with immersion in 20% alcoholic formalin, following perfusion of the animal with 10% formalin.

Each of the five stains used in this histopathological study, Hematoxylin-Eosin, Luxol Fast Blue, Oil Red O, Cajal's Gold Sublimate, and Hortega's Silver (modified), contributed some evidence of change or reaction in the tissue. Of these five, Cajal's was the best and most sensitive index in every phase of damage and healing.

Silver showed the most pronounced pathology of any metal used, while stainless steel and gold were the least harmful to brain tissue.

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Clinical Specimens and Reference Samples for the Quality Control of Laboratory Accuracy*

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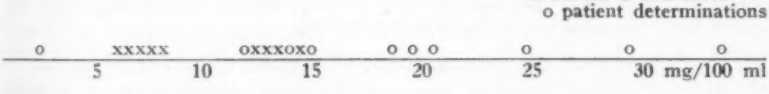
Several years ago, two of the authors proposed using the values of the clinical specimens as a partial means of detecting errors in clinical laboratory procedures (5). To the authors' knowledge, use of clinical specimens has not received much attention. Most laboratories operating a quality control program rely exclusively on testing standard samples (3). More recently, pooled serum or plasma with known concentrations of constituents that are stable over long periods of time have been employed. In this paper we suggest that both standard or reference samples as well as clinical values have a place in the control program. Each is valuable in its own way and one should complement the other. First, however, consider the basic principles of laboratory control.

Principles of Laboratory Control

The basis of the method used for detecting change in a laboratory procedure is "unexplained variation." For example, if we selected a substance known to contain 10 mg/100 ml and ran tests on 10 aliquots, we might accumulate the following data: (Each cross on the chart below represents 1 determination).



If this same substance exists in blood, and 10 different patient specimens were tested we would obtain much more widely scattered values, illustrated by the small circles on the above chart. In addition to the unexplained variation, patients differ from one another. Their diseases may also affect the substance in question; hence, it is reasonable to expect a much more widely scattered set of values for the patient specimens.

The statistical tool used for describing variability, regardless of whether we are discussing a standard substance or clinical tests, is the

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standard deviation. This is defined to be

$$s = \left\{ \begin{matrix} \text{standard deviation} \\ \text{of a single determination} \end{matrix} \right\} = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$$

In words, it is the sum of the squares of the differences of the measurements from their mean, divided by one less than the number of measurements. The square root of the result is then taken. The standard deviation of the mean of a group of determinations can be shown to be[1]

$$s_{\bar{x}} = \left\{ \begin{matrix} \text{standard deviation of the} \\ \text{mean of a group of tests} \end{matrix} \right\} = \frac{s}{\sqrt{n}}$$

In words, it is the standard deviation of a single test divided by the square root of the number of tests which go into the mean.

The above is basic statistical theory, regardless of the manner in which it is used. In terms of our problem of laboratory control, the most important feature of the standard deviation of the mean is the fact that *we can make it as small as we please by increasing n*, the number of determinations in the sample. We are now ready to consider one method of approach to using clinical specimen values for laboratory control.

Using Clinical Values in Terms of an Arbitrary Error

The problem that we consider is this. For a given laboratory test, we decide in advance the magnitude of an error we wish our control chart to detect for us. We must also specify a probability of being wrong, i.e. of suspecting a laboratory error when in fact none exists. We also need, from previous clinical experience *(When the laboratory is known to be operating without major error)* the clinical average and the standard deviation of a single test. We now put all of these into the following formula[2] and solve for n. This gives us the number of clinical values to average for control purposes.

$$n = \left[\frac{ts}{e} \right]^2$$

Where t is the abscissa of the normal curve, specified when we choose our odds of being wrong.

s is the standard deviation of a single clinical specimen.

e is the error we choose to detect, i.e. an erroneous shift of the clinical mean.

Some Examples

Hemoglobin: Suppose we wish to detect an erroneous shift of 0.5 gm/100 ml of whole blood. From previous experience we know the

[1]For proof, refer to a book on statistical theory, for example (2).

[2]This is simply a rearrangement of $t = \frac{\bar{x} - m}{s/\sqrt{n}}$ where $e = \bar{x} - m$, the error to be detected, and m is the clinical population mean. The source of this formula may be found in reference (2).

standard deviation of our clinical values to be 2.5 gm. Arbitrarily, we choose our odds of being wrong to be 5%, which determines t to be 1.96, from a table of the normal curve found in a book on statistics, for example (2). Thus,

$$n = \left[\frac{(1.96)\ (2.5)}{0.5}\right]^2$$
$$= [9.80]^2$$
$$= 96$$

Hence to detect an erroneous shift of 0.5 gm. in the hemoglobins we should average about 100 clinical specimens for each point on the control chart. It took less than 3 minutes for one of us to compute the average of 100 specimens using an electric adding machine. With practice, par should be about 2 minutes.

To illustrate our theory with some actual laboratory data, we have obtained hemoglobin data from two different laboratories. This was accomplished by taking the laboratory records and computing the average for consecutive sets of 50 determinations. Since standardization data were not available, we arbitrarily used the earliest 500 determinations for estimating the over-all mean and standard deviation. The results are as follows:

Laboratory	Mean	Standard Deviation	Standard Deviation of Mean of 50
Number 1	13.84	1.97	0.278 gm/100 ml whole blood
Number 2	12.40	2.31	0.327 " " " " "

The results of all of the above are plotted on the charts shown in figures 1 and 2, one chart for each laboratory. A hemoglobin scale appears at the left side of each chart. Slightly to the right of each scale, the mean of the first 500 tests is shown along with a vertical line representing ± two standard deviations of the mean of 50 determinations. Each point on the chart is the mean of 50 clinical tests. The dates on which the averages were computed are shown along the bottom of the charts.

Several interesting features can be seen by study of figure 1. (a) Either some unusually high values were included in the first 500 determinations, or a change occurred in the laboratory shortly after this. (b) For several months, until about April of 1960, no discernable trend is noted. (c) Beginning in May, periodicities are seen with the 4 most recent averages the lowest of those recorded. In fact they are nearly 8 standard deviations below the average of the first 500 determinations.

The hemoglobin averages from laboratory 2 shown on figure 2 also show some interesting features. Their variability is greater than that seen in figure 1, and though not so marked, some trends do seem to exist. Note that in some periods, a substantial proportion of the averages fall below the two standard deviation limits.

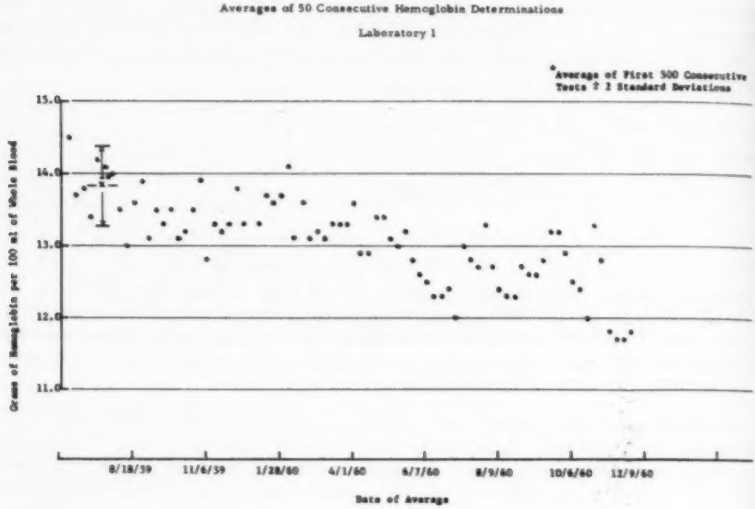

Figure 1

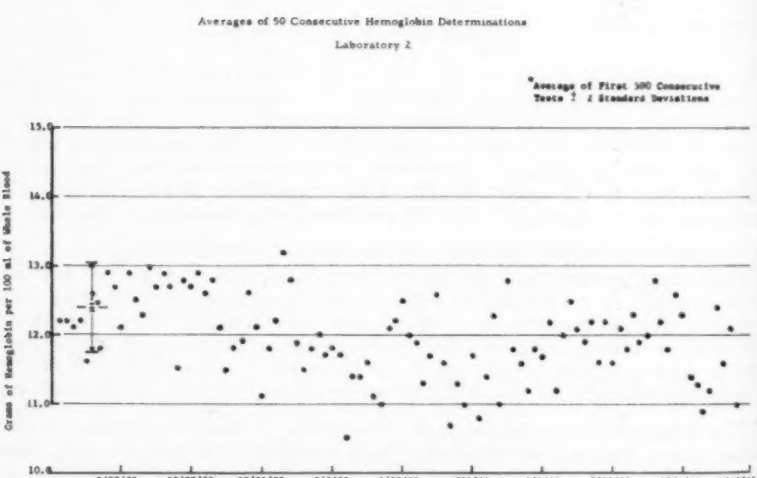

Figure 2

Blood Sugar

Using clinical specimens for obtaining information about fasting blood sugar determinations presents a problem not present in the case of hemoglobins. High values for a blood sugar measurement are not uncommon, but correspondingly very low values i.e. below 50 mg/100 ml are rarely seen. The result is that distributions of clinical blood sugar values are "lopsided." From the point of view of using clinical specimens for control purposes, considerable numbers of clinical specimens should be used in each average. In our example, we used 100 specimens in each average, but we have not made a thorough study of an optimum number to use.

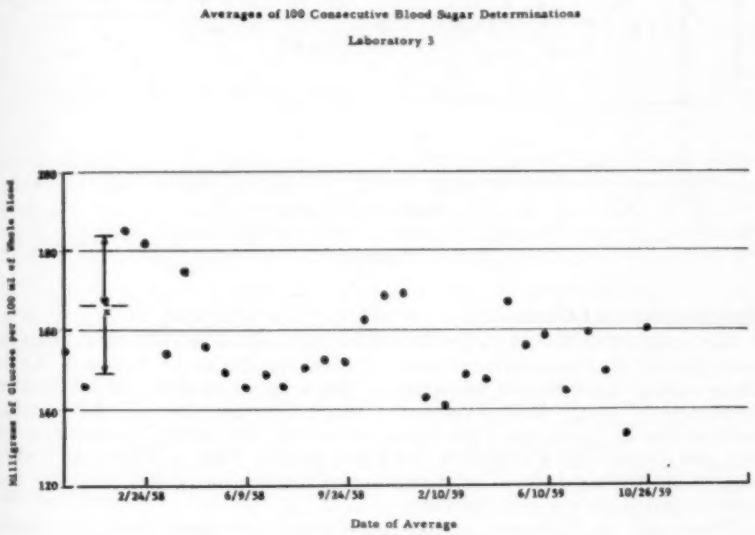

Figure 3

From a third laboratory we have obtained data on blood sugars and have prepared figure 3 in a manner similar to figures 1 and 2. In this case, however, each average is based on 100 consecutive blood sugar determinations excluding postprandial and glucose-tolerance tests. Of the 3 charts prepared from laboratory data, this one shows the least tendency to stray outside of the $\pm$ 2 standard deviation limits. Some trends are noted, however, and more points than one would expect on a random basis do fall outside the limits.

We have seen trends on all the charts and on all have seen many points fall outside the $\pm$ 2 standard deviation limits. The question arises as to how much of this may be due to random, inconsequential effects and how much may be due to correctable laboratory error. To provide some information about this question, we have prepared a "Random Normal Chart."

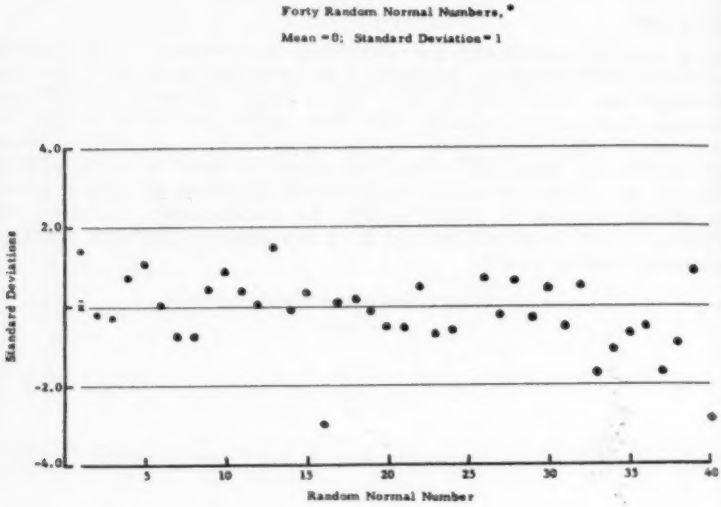

Figure 4

Random Normal Chart

We took an arbitrary point in a table of random normal deviates (1), and plotted 40 consecutive values of them as shown in figure 4. All of these values came from a population with a mean of zero and a standard deviation of unity. Hence on the average, 2½ percent will fall outside each of the ± 2 standard deviation limits. In fact, only 2 points (out of 40) are outside ± 2 standard deviation limits. This is what one would expect, on the average. The exception of the two large deviate numbers here is that both are low numbers.

The point is that any trends in clinical specimen averages which are of the order of those seen in chart 4, i.e. within ± 2 standard deviations probably do not reflect any real change in the laboratory. Also, if a laboratory is reporting results with high precision and accuracy, their averages will stay within the limits chosen. For example, if ± 2 standard deviation limits are used, 95 percent of the averages will remain within the limits, provided there is no "seasonal" change; otherwise this must be taken into account.

Direct Comparison of the Clinical and Control Sample Approach

In the previous section we discussed and illustrated how the clinical values can be used to detect an error whose magnitude is arbitrarily determined. Let us now show how a direct comparison may be made of the control method based on reference samples and that based on clinical values. There are two things we need for our comparison:

*Dixon, W. J. and Massey, F. J. Introduction to Statistical Analysis. McGraw-Hill Book Co. 1957.

1. s_c, the standard deviation of the clinical specimens, determined when the laboratory is known to be operating without major error.

2. s_e, the standard deviation of the reference samples.

Both of the above standard deviations are constants determined by each laboratory for a given test.

Since the standard deviation of a mean is $s/\sqrt{n}$, by increasing n, we may make the standard deviation of the mean of the clinical specimens as small as the standard deviation of the control specimens. We do this simply by setting the ratio of the two standard deviations equal to unity and solve for n.

$$1 = \frac{s_c/\sqrt{n}}{s_e}$$

$$n = \frac{s_c^2}{s_e^2}$$

In words, if we wish to set up control charts on clinical specimens whose "error detecting" ability is the same as that of the reference samples, simply divide the variance (square of the standard deviation) of the clinical specimens by the variance of the standard specimens. The result is the number of clinical specimens to use in each average.

The preceding discussion is based on the assumption that for the routine control program, only single reference samples are run. If two reference samples are run, then the above expression becomes

$$n = \frac{2s_c^2}{s_e^2}$$

and the number of clinical specimens would be doubled.

An Example

Hemoglobin: Suppose, as in the previous theoretical example, our standard deviation of the clinical specimens is 2.5 gm/100 ml, and furthermore assume that the standard deviation of the reference sample is 0.2 gm/100 ml.
Then

$$n = \frac{(2.5)^2}{(0.2)^2}$$

$$n = 156.$$

In other words, we would have to add and average 156 clinical specimens to achieve the same "error detecting" ability as would be provided by a single reference sample. This may seem like a large number of clinical specimens but when the total amount of work is considered there are definite advantages to using clinical specimens. Actually both clinical and reference samples have an important function which we will discuss in the next section.

Discussion

We have compared, on a statistical basis, the relative merits of clinical and reference samples for the purpose of laboratory control of error. We illustrated our method with data from 3 laboratories, but we have not said much about error itself. What kinds of errors are there and to what extent can each kind be detected? We arbitrarily classify them into two kinds; (1) random errors and (2) constant errors. Random errors are those which, by definition, occur only on a sporadic basis. They consist of, for example, erroneously reading a measuring device, or a mistake in a computation. They affect only the determination of single laboratory specimens. Constant errors are those which affect large numbers of tests, for example, they may be caused by a lightbulb which has become faulty in a colorimeter or an improperly prepared stock solution of a reagent used in a test.

Random errors are almost impossible to detect with either the use of clinical specimens or reference samples. In fact, if a random error were made when testing a reference sample, it would probably lead to a fruitless search, because search would be made for a constant error. A single or a few random errors would not likely be detected by using the clinincal values either, because their numerical weight would be small when combined with a hundred or more accurate clinical values. So neither method is of much value for detecting random errors. The best method for guarding against these errors is to have competent technologists reporting accurate and precise results.

Constant errors are likely to be detected equally well assuming equal "error detecting" ability and assuming the constant error is present in all clinical values. With the clinical specimen method, however, we must wait until a hundred or so measurements have been run before the error is detected. On the other hand, a reference sample may not be run at the time a constant error first made its appearance. Thus there appears, from the error-detecting point of view, to be no great advantage to using either clinical or reference sample specimens. We saw from actual laboratory data that trends may also develop. We see no great difference between either the clinical or reference sample method for detecting real trends.

In actual practice, it seems most desirable to use both methods. For example in the control of hemoglobin measurements, average the clinical specimens routinely as one method of control; then at periodic intervals run a series of standard or reference samples as a further check. A standard or reference sample should be run immediately when a clinical average goes beyond the control limits.

For hemoglobins, the cyanmethemoglobin method provides a stable hemoglobin standard or reference sample that may be checked at regular intervals (4). Blood sugars and several other clinical chemical measurements also provide examples of tests with readily available stable standard or reference control samples. These are prepared in a protein solution (serum) and may be measured with each set of analyses.

Summary

An additional level of quality control in the clinical laboratory has been reviewed. Since it requires clinical specimens there is no increase in work load because these values are accumulated in the course of daily laboratory activities.

The use of clinical specimens has been compared with reference samples for quality control. Both may fail to detect a random error and appear to be equally sensitive to detect a constant error, although the numbers of clinical specimens employed may preclude prompt recognition.

The use of data obtained from clinical specimens is recommended to supplement and complement other methods of quality control employing standard and reference samples.

Thanks are due to Mrs. Donna Bartlett for accurately and quickly tabulating the thousands of laboratory tests, the averages of which are included in this paper.

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SEASONS GREETINGS

Infectious Bacterial Aerosol from Dropped Petri Dish Cultures

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I. Introduction

Previous investigations have shown that most laboratory-acquired infections are caused by unrecognized microbial aerosols created during an accident or during routine laboratory procedures.[2] Infection of five laboratory workers, after the dropping of petri dish cultures of *Pasteurella tularensis,* prompted investigation of the extent of aerosol formed during such an accident. The petri dishes were dropped in the hallway of an infectious disease building. At the time of the accident or during the cleanup, 14 persons were present or entered the hall from adjoining laboratory rooms. All previously had been vaccinated with a non-viable tularemia vaccine. Of the five tularemia infections one was pulmonic, two typhoidal, and two were subclinical. The subclinical infections were diagnosed on the basis of a rise in blood agglutination titers. One person infected was 70 feet from the accident site.

The accident was re-enacted with a non-pathogenic organism to determine probable concentrations of bacterial aerosol at the site of the accident and in the surrounding area. In addition, laboratory experiments were done to determine if bacterial aerosols were released when plastic and glass petri dishes were dropped.

II. Methods

A. RE-ENACTED ACCIDENT

Re-enactment of the accident was carried out at the site (see Figure 1). An effort was made to follow closely the sequence of events which were revealed by the accident investigation.

Two one-half gallon ice-cream cartons (without lids), each containing ten glass petri dishes inoculated with *Serratia marcescens* (100-1,000 colonies per plate on Bacto-nutrient agar) were pushed from the shelf of an autoclave, and allowed to fall 40 inches onto a concrete floor. Then the dishes and boxes were covered with towels that had been soaked in a 5,000 parts per million solution of sodium hypochlorite. The area was sprayed with the same solution from a three-gallon pressure sprayer. The petri dishes covered by the towels were picked up and placed in an autoclave after 30 minutes. Then the floor of the hallway was sprayed with hypochlorite solution.

Mechanically timed, one hour capacity, air slit samplers[1] that rotated an agar plate under the impinging slit once in 60 minutes and sampled one cubic foot of air per minute were used to determine the amount of

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bacterial aerosol created by: (1) the dropping, opening and breaking of petri dishes, (2) the decontamination procedure, and (3) the cleanup of debris. The number of *S. marcescens* colonies recovered during each portion of the hour was determined by dividing the surface of the agar plate into time zones. Samples of air collected at each sampling station immediately before each test assured the absence of *S. marcescens* organisms. Tracer smoke was used to determine the path of air flow in the hall and in adjoining laboratory rooms. The procedure was re-enacted eight times with variations.

B. LABORATORY EXPERIMENTS

Laboratory experiments were designed to compare glass petri dishes with plastic petri dishes to: (1) determine the bacterial aerosol produced during the dropping and/or breakage, (2) verify results obtained in the re-enactment of the accident, (3) determine the bacterial aerosol produced with other organisms.

To control air movement and to prevent infection of personnel, the laboratory experiments were conducted in a bacteriological cabinet system.[2] Four different species tested were: *Bacillus anthracis, Staphylococcus aureus, Escherichia coli B.*, and *Serratia marcescens.* For each organism, ten plate drops were made with each of 1, 5, 10 and 20 plates per drop, for glass petri dishes and then for plastic petri dishes. The petri dishes were prepared, using standard bacteriological procedures, to obtain 200-300 colonies per plate. Ten drops were made for each group of dishes (1, 5, 10 or 20) by placing the inverted dishes on a shelf (Figure 2), then pulling the dishes off the shelf and allowing them to fall 24 inches to the stainless steel floor of the cabinet. Air slit samplers of five minute sampling capacity, that revolve a 90 millimeter diameter agar filled glass petri plate under the slit once in five minutes, sampling one cubic foot of air per minute, were used as the sampling device. Immediately preceding the dropping of the dishes, five minute control samples were taken at each sampling location in the cabinet to determine the presence or absence of contamination within the cabinet. A five minute sampling period was maintained, starting immediately prior to the plate drop and ending prior to cleanup of debris. After each test, the debris from the completed drop was gathered and passed into the autoclave. Following the cleanup, ultraviolet germicidal lamps in the cabinet system were turned on, and a 20 minute air purge was maintained before control samples were taken for the next test. In addition, the slits of the samplers were decontaminated by washing them with 95 percent ethyl alcohol.

The average time of air flow through the cabinet system was 24 seconds, and the rate of smoke flow through the cabinet was six inches per second. After completing the work with each organism, the cabinet was decontaminated with a 3:5 methanol-formaldehyde mixture vaporized with the aid of steam.

III. Results

A. RE-ENACTED ACCIDENT

The air movement* in the laboratory building is shown in Figure 1. Air turbulence was detected above the floor near the drop area, and this turbulence decreased near the north end of the hall. Control air samples immediately preceding the test showed no *S. marcescens*.

Repeated drops of glass petri dishes showed that *S. marcescens* could be recovered at all sampling locations during and after the simulated accident, decontamination procedures, and cleanup of debris (Table 1).

Figure—1 BUILDING LAYOUT-ACCIDENT SITE*

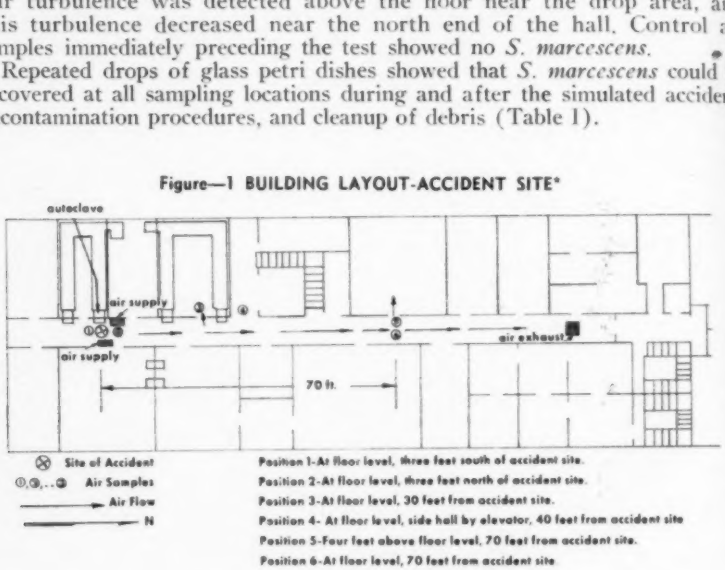

autoclave
air supply
air supply
air exhaust
70 ft.
Site of Accident
Air Samples
Air Flow
N
Position 1-At floor level, three feet south of accident site.
Position 2-At floor level, three feet north of accident site.
Position 3-At floor level, 30 feet from accident site.
Position 4- At floor level, side hall by elevator, 40 feet from accident site
Position 5-Four feet above floor level, 70 feet from accident site.
Position 6-At floor level, 70 feet from accident site

TABLE 1

Accident Re-enactment

Sampling Station	AVERAGE NUMBER OF COLONIES ON AIR SAMPLER DISHES Glass Petri Dishes				Plastic Petri Dishes After Dropping	
	After Dropping		Decontamination			
	First 20 Min. After Drop	20–50 Min. After Drop	First 15 Min. After Drop	During 30 Min. Decontamination Dish Pick-up*	First 20 Min. After Drop	20–50 Min. After Drop
1.....	8	0	7	20	1	0
2.....	13	4	23	61	5	0
3.....	15	1	0	2	0	0
4.....	9	16	0	1	0	0
5.....	6	3	3	6	0	0
6.....	1	1	3	12	0	0

† 15 minutes after dropping dishes, decontamination was done; dishes were picked up at the 30th minute, (average of three tests).

* U. S. Army Photograph

† The ventilation system in this building is non-recirculating.

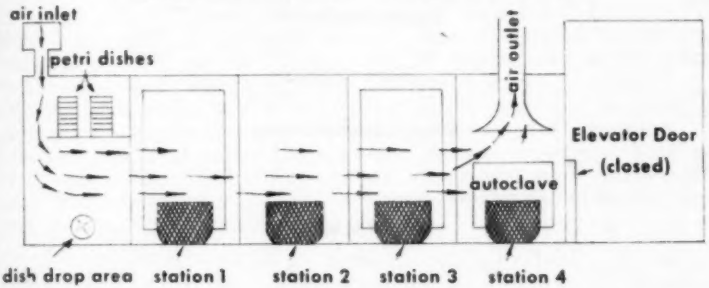
In the final two tests at the accident site polystyrene culture dishes were substituted for the standard glass petri dishes to determine if non-breakable dishes influenced the amount of bacterial aerosol produced. The results (Table I) showed that less bacterial aerosol was produced when polystyrene culture dishes were dropped.

B. LABORATORY EXPERIMENTS

The data on bacterial aerosol produced from dropped petri dish cultures are summarized in Table II. The results represents the sum of all samples from all four sampling locations (Figure 2). The dropping of glass dishes produced a greater aerosol than the dropping of plastic dishes in all of the tests except when five plates were used with *Serratia marcescens* and *Bacillus anthracis*.

Figure 2*

BACTERIOLOGICAL DOUBLE-SIDED AIR-TIGHT CABINET SYSTEM
(for details see Figure 2 in Reference 2)



Variation in data necessitated the application of the analysis of variance technique to determine the significance of the variation. When this analytical technique was used, "Mean Counts," or best estimates overall plate drops and sampling stations, gave a ratio of bacterial aerosol for glass petri dishes to plastic petri dishes of 3:1 for *Bacillus anthracis*, 22:1 for *Staphylococcus aureus*, 24:1 for *Serratia marcescens*, and 69:1 for *Escherichia coli-B*. Considering only the ten plate drop, differences are of even greater magnitude as follows:

MEAN COUNTS
(Square of Mean Square Root)
Ten Plate Drop

	Serratia marcescens	Escherichia coli-B	Bacillus anthracis	Staphylococcus aureus
Glass Petri Dish	5.0	17.8	0.32	2.04
Plastic Petri Dish	0.17	0.04	0.006	0.029

* U. S. Army Photograph

Tests differed significantly within each type of plate, number dropped and organisms. However, data are not adapted to precisely determining reproducibility, since they were collected over a long time and the experiment was not designed to determine such sources of variation as day to day, technician, test equipment, etc.

In order to use the analysis of variance technique, the raw data were transformed to square roots, and counts of 350 were substituted for the four instances of "TNTC"* obtained in experimental results for *S. marcescens*. The results of the analysis of variances support the conclusion that a greater bacterial aerosol is created by dropping glass petri dishes. This difference between the aerosol created by the two types of petri dishes proved to be highly significant—much beyond the one percent level needed for significance in each of the series of tests with all of the four organisms tested.

TABLE II
BACTERIAL AEROSOL FROM DROPPED PETRI DISHES

	Dishes Per Test			
Dishes	1	5	10	20	TOTAL*
		Serratia marcescens		
Glass	8	189	591	221	1009
Plastic	0	556	25	11	592
		Escherichia coli-B		
Glass	35	338	910	2873	4156
Plastic	1	38	9	87	135
		Bacillus anthracis		
Glass	6	12	39	29	86
Plastic	1	37	1	2	41
		Staphylococcus aureus		
Glass	34	65	145	600	844
Plastic	15	3	8	137	163

*Number of colonies recovered.

IV. SUMMARY

An accident involving the dropping of 20 agar plates with surface colonies of *Pasteurella tularensis* caused 5 of 14 exposed laboratory workers to acquire clinical or subclinical tularemia.

A study of the re-enacted accident, carried out by sampling air in various locations after plates containing colonies of *Serratia marcescens* were dropped, indicates that aerosols were produced by (a) rebound or breakage of the plates, and (b) decontamination with a spray apparatus and picking up the scattered and/or broken petri dishes. Bacteria were carried by air currents to distances remote from the accident site.

Bacterial cultures in unbreakable plastic petri dishes released less bacterial aerosol when dropped, than those in glass petri dishes.

The amount of bacterial aerosol increased with an increase in the number of plastic or glass petri dishes that were dropped.

The amount of bacterial aerosol produced varied for different organisms tested.

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*Too numerous to count.

Evaluation of Renal Tubular Function Using a Simple Kinetic Method *[1,2,3]

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INTRODUCTION

Since the establishment of renal clearance determinations using p-aminohippuric acid by Smith,[7] many attempts have been made to avoid the use of intravenous constant infusion methods. Many simplified techniques using single injections of p-aminohippuric acid (PAH) have been described in the literature and have included single injections by intravenous, intramuscular, and subcutaneous routes.

Smith's method for renal clearance has been verified many times and is generally used for evaluation of the functional status of the kidney. With few exceptions, most of the work published on simplification of this method has attempted to establish clearance values comparable to those reported by Smith[7,8] and others.

In 1949 Tacket[9] used a single injection technique, and measured plasma PAH levels. He related falling plasma concentrations of PAH to removal of PAH by the kidneys, and attempted to correlate results with the standard clearance values without complete success.

Newman[5] showed in 1949 that plasma concentrations of PAH can be described accurately after a single injection of PAH since they followed a consistent curve when plasma concentrations and time were plotted on semilogarithmic graph paper. Clearances were calculated by using the urine and plasma PAH concentrations at the mid-time of the falling plasma concentration.

In 1955 Clausen[2] described a new clearance method called the "clearance speed" using PAH as a test substance. "Clearance speed" was defined as the time required after injection of a known amount of PAH for the blood concentration to decrease to one-half of this value. The actual PAH concentrations were not important, only the ratio between the two blood concentrations. Two blood levels were plotted against time on semi-log graph paper; any point on the line was chosen and its half value was found further below. The distance between these two points was expressed as the "clearance speed." This method does not attempt to correlate clearance with the older clearance values established by Smith,[7,8] and others, but represents a new approach in renal clearance evaluation.

Elliott and Wu[3] in 1957, in their work with benzoate and hippurate treated excretion data on the basis of first-order kinetics to obtain an excretion constant for the removal of the respective substances from the body.

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*Kimble Award Winner.

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2. Part of the material contained in this paper was taken from a thesis submitted by C. RAY RATLIFF to the faculty of Birmingham-Southern College in partial fulfilment of the requirements for the degree of Master of Science.
3. Presented in part before the Joint Meeting of the Alabama Association of Pathologists and Alabama State Society of Medical Technologists, May 14, 1960, Birmingham, Alabama.

The controversial literature concerning attempts to correlate falling plasma level determination (single injections) with established clearance values seems to indicate that a new approach to evaluation of renal function is in order. The primary purpose of these experiments has been to establish a simplified technique for measurement of renal tubular function using PAH (p-aminohippuric acid) and based on a kinetic interpretation of urinary excretion data. It is hoped such measurements can be made by those medical facilities unable to apply the presently available clearance methods.

The following experiments and data are presented to show how a kinetic interpretation of excretion data may be the solution to the problems associated with study of renal function following single injections of a test substance.

METHODS

Patients with suspected or known renal disease were selected for obtaining data on abnormal kidney function. Clearances using a constant infusion of PAH and inulin were performed on each subject. The clearance methods described by Smith[8] were used in this study. In addition to these recognized clearance procedures, excretion experiments using a single intravenous injection of PAH were performed on each subject.

The hospitalized patients were fasting on the morning of the tests and were given 1 liter of water to drink one hour before commencing the procedures. An indwelling catheter was used to maintain the greatest accuracy in collection of the urine samples. The patients remained in bed throughout the experiments. Just prior to injecting the test substances, the bladder was emptied and a urine specimen was collected as the urine blank for use in the analytical procedures. In addition, a blood sample was withdrawn in a heparinized syringe for the plasma blank in the analyses.

The test substances were then administered as follows: At zero time an injection of 2 cc. of 20% p-aminohippuric acid (PAH) (Sharp and Dohme, Phila., Pa.) was given intravenously. The injection was completed within 30 seconds and the time noted at its completion. Six samples of urine were collected at intervals of 10 minutes. The exact time of collection and volume were recorded for each specimen. Urine specimens were measured in a graduate cylinder and aliquots taken for analysis.

At the end of this collection period, a priming injection consisting of 3 cc. of 20% PAH and 20 cc. of 10% inulin (U.S. Standard Products Co., Mount Prospect, Ill.) was given intravenously. Immediately after this a maintenance solution composed of 5 cc. of 20% PAH and 30 cc. of 10% inulin in 500 cc. of isotonic saline was given intravenously at the rate of 60 drops per minute. Sterile technique was observed in the handling of all injection material and equipment.

After 30 to 40 minutes to allow equilibration, the bladder urine was discarded and heparinized blood samples and urine samples were collected at 10 minute intervals for a total time of 40 minutes. The bladder

was washed with 30 cc. of isotonic saline and air at each collection period to insure accuracy. Aliquots were placed in test tubes for subsequent analysis. Enough blood should be withdrawn to provide 2 cc. of plasma for the analysis.

The "standard" clearance with PAH and inulin were calculated by the usual formula:

$$C = \frac{UV}{P}$$

C = clearance of substance in milliliters per minute.

U = concentration of substance in the urine in milligrams per 100 ml.

P = concentration of substance in plasma in milligrams per 100 ml.

V = volume of urine excreted per minute.

A group of active people in apparent good health with no known renal disease was selected to provide excretion data using a single injection of 2 cc. of 20% PAH (400 mg.). Members of this group, an equal number of male and female subjects, were primed with 6 to 8 glasses of water by mouth prior to the test and were fasting. With this group, voided urine samples were collected with complete emptying of the bladder every 5 to 10 minutes for a total time of 60 minutes. The collection times and urine volumes were recorded. Since the subjects were hydrated before and during the experiment, all of the subjects adapted readily to these frequent micturitions. The average urine flow was from 8 to 12 cc. per minute. Specimens from the male were collected in a graduate cylinder; specimens from females were collected in a small basin after which they were transferred to a graduate cylinder for measurement.

Plasma and urine PAH concentrations were determined by Smith's modification[7] of the method of Bratton and Marshall. Inulin was determined by the direct resorcinol method of Schreiner.[6] The urine samples from the "normal" group were treated also by the method described by Newman[5] in determining PAH and conjugated PAH as "free" and total PAH. These values were determined in order to study the effects, if any, on the resultant excretion data. In these experiments, the time of hydrolysis was increased from 90 to 180 minutes on duplicate samples in order to check completion of reaction.

In addition to these analyses, the quantity of uric acid in each of the "normal" urine samples was determined by the method of Caraway[1] and plotted as cumulative endogenous uric acid excretion. This was done to study the nature of the curve as an indication of collection accuracy in the sampling method for voided urine since endogenous urate is known to be excreted at a constant rate.

Plasma and urine samples were stored at 0° C. until all analyses were completed.

All analyses were run in duplicate, and colorimetric procedures were adapted to the Coleman Junior Spectrophotometer.

The First-Order Kinetics In Interpretation Of the PAH Excretion Data

The interpretation of the PAH excretion data in the single injection investigations is based on first-order kinetics where an excretion constant can be determined from the amount of PAH in the body and the amount excreted by the kidneys per unit time. The application of this principle to renal dynamics may be shown as follows:

$$H_o \xrightarrow{K_E} E$$

amount PAH in body	excretion constant	amount PAH excreted

The amount of PAH removed from the blood by the kidneys is proportional to the body load (400 mg. at zero time) and the rate of excretion by the kidney. Thus the functional capacity of the kidney can be determined when the body load and the amount excreted are known. The excretion constant (K_E) is then related to the kidney's ability to excrete when subjected to these conditions and can be derived as shown by Wu and Elliott.[10]

The procedure for calculating the excretion constant (K_E PAH) in these single injection experiments is as follows:

H_o equals the amount of PAH administered at zero time in milligrams; E represents the cumulative excretion of PAH in milligrams;

$\frac{E}{H_o}$ represents the fraction of dose excreted each period; and

$(1 - \frac{E}{H_o})$ equals the amount of PAH remaining in the body. E, the cumulative amount of PAH excreted in milligrams, is calculated by totaling the milligrams of PAH for each successive period of time. The ratio of $\frac{E}{H_o}$ is determined by obtaining the quotient of these cumulative amounts and the dose of 400 milligrams (H_o). The fraction $(1 - \frac{E}{H_o})$ is then determined for each of the $\frac{E}{H_o}$ ratios. These data were plotted on semilogarithmic paper, plotting the fractions $(1 - \frac{E}{H_o})$ on the semilogarithmic axis and the time on the other axis. K_E PAH is then determined by measuring the time for the plotted fractions to pass a point of one-half its original value, i.e., the point at which the 200 milligram (half-dose) line intersects the plotted curve and read as time in minutes. This

is T½. Then:

$$1.\ K_E = \frac{1\ n\ 2}{T½}$$

$$2.\ K_E = \frac{0.693}{T½}$$

$$3.\ K_E = \frac{0.693 \times 60}{T½}\ *$$

RESULTS

(A) Results Obtained on Single Injection Experiments with "Normal" Individuals.

The values for K_E PAH on single injection experiments range from 1.05 to 3.50 hr^{-1} with an average of 2.30 hr^{-1}, and a standard deviation of 0.26. These data are shown in Table I, and it can be seen that results in male and female subjects are such as to indicate no significant distribution or variation with regard to the sexes.

The lowest value of K_E PAH (1.05 obtained on a male) was 0.65 units lower than the next lowest value of 1.70. As shown in Table II 91 per cent of the "normal" values were significantly higher than the K_E PAH values found on the "abnormal" group.

In Fig. 1, these differences of K_E PAH are illustrated by a plot of the average values for the "normal" and "abnormal" groups. Some slopes on the "abnormal" subjects (0.22, 0.24, 0.60, etc.) were extremely slight and had to be extrapolated over several sheets of graph paper before T½ could be determined. A comparable difference in the time for half-dose excretion; 18.5 minutes for "normals" and 56 minutes for "abnormals" can be seen (Fig. 1).

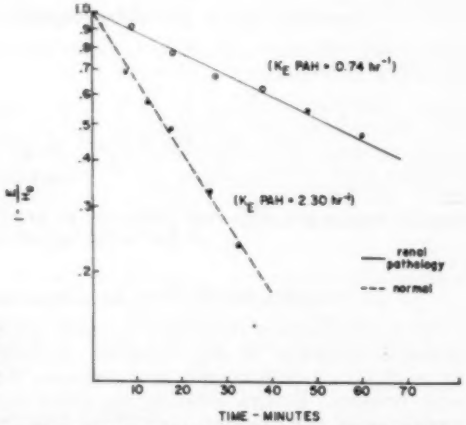

Figure 1. Average values of "normal" and "abnormal" K_E PAH showing the distinctive differences found in the two groups after injections of PAH.

*These equations are mathematical summations of the first-order reaction applicable to these data. In equation 1., n 1 x 2 represents the natural logarithm multiplied by two. In equation 3., 60 represents time in minutes. It may be seen that equation 3. will resolve to $\frac{41.6}{T½}$. T½ is obtained from the value (time in minutes) noted where the half dose (200 mg) axis intersects the time in min. axis.

For a more thorough explanation of mathematical treatment of such type data, the reader is referred to H. Wu and H. C. Elliott (cited in references). The fundamentals of first-order reactions may be obtained from a physical chemistry text such as: Getman and Daniels, Outlines of Physical Chemistry, John Wiley & Sons, New York, 1946, p. 345-349.

Observations On PAH and Conjugated PAH

Newman[5] studied the effects of metabolic alteration of PAH in the body on his clearance data with single injection experiments. Single injection experiments, resulting in a falling plasma concentration, yielded an increasing proportion of conjugated PAH (p-acetylaminohippuric acid) to free PAH in the urine, and the clearance of conjugated PAH (PACA) became higher than the clearance of PAH.

Determination of total PAH in this investigation was made from the urine samples of various subjects in the "normal" group. A difference was realized between the quantity of PAH excreted, *per se*, and the total excreted PAH (PAH + PACA) after analysis by Newman's[5] method and calculation of K_E PAH. A difference of 0.50 units was noted between the average K_E PAH and K_E PACA results as shown in Fig. 2. Furthermore, the ratio of PACA to PAH in the urine increased directly with time during the excretion experiments as had been reported previously by Newman[6] in 1949.

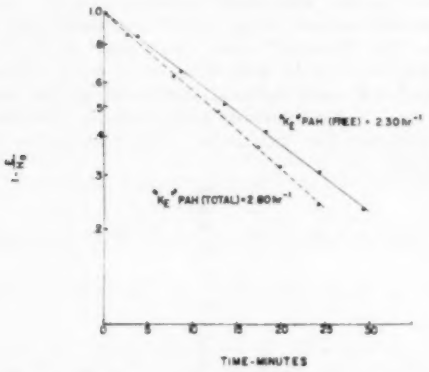

Figure 2. Average plot of K_E PAH (FREE) and K_E PAH (TOTAL) found after single injections of PAH in the "normal" group.

Results With Uric Acid and Sampling Methods

All urine samples from the "normal" group were voided every 5 to 10 minutes. Accuracy of this collection method, i.e., by voiding, was studied by analysis of each sample to determine the amount of endogenous uric acid present. Errors relating to urine collection such as incomplete emptying of the bladder, poor urine flow, or incorrect volume measurements may be reflected by the nature of the cumulative uric acid excretion curve. These results indicate that voided urine specimens may be used satisfactorily, omitting catheterization. The subjects in this group were aware of the necessity for complete sampling and it is felt this "cooperation factor" contributed to good results. Fig. 3 in which the quantity of cumulative uric acid in milligrams is plotted against time

illustrates quite clearly the linear relationship possible when samples are complete and timing is accurate.

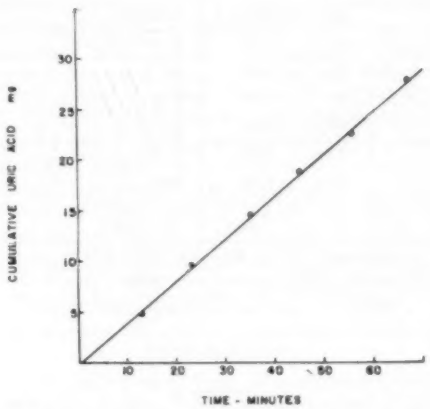

Figure 3. Plot of cumulative uric acid on a "normal" subject. Analysis was done on samples collected during the single injection experiment with PAH. The linear relationship is shown when collection of samples are accurate by free-voiding methods.

(B) Results Obtained With Subjects Having Renal Disease.

The data obtained on PAH clearance, inulin clearance, filtration fraction, and single injection excretion as K_E PAH are shown in Table II.

The values obtained for these tests were all in the "abnormal" range except the PAH clearance (C_{PAH}) on one subject designated as "W.H." This value is questionable in view of the low values obtained for inulin clearance (C_{Inulin}), filtration fraction (F.F.) and K_E PAH. Two subjects with abnormal values for PAH and inulin clearances had K_E PAH values (M.B. = 1.08, and O.L = 1.40) which fell within the lower range as determined on the "normal" subjects.

Although the size of this group is limited, a significant relationship between impaired renal function and single injection (K_E PAH) is evident (Fig. 1). All of the various renal conditions have not been explored at this time and a more thorough study should give more significant results.

DISCUSSION

The primary purpose of these experiments has been to establish a simplified technique for measurement of renal function, based on a kinetic interpretation of excretion data. The establishment of such a method of measurement of renal function would provide a technique within the reach of those unable to apply the presently available methods such as is now the case in some medical facilities.

TABLE I

Results with single injection PAH excretion as K_E PAH on the "normal" group

MALE			FEMALE		
Subject	Age	K_E PAH	Subject	Age	K_E PAH
R.R.	33	1.70	E.R.	29	2.30
C.C.	24	3.00	J.F.	23	2.00
R.B.	36	2.90	C.E.	35	2.30
H.L.	27	1.05	B.C.	22	1.90
C.R.	22	2.20	N.R.	22	2.50
H.E.	34	3.50	M.T.	36	2.40

	Age	K_E PAH
Average	28	2.30
Std. Dev.	5.0	0.263
Range	22–36	1.05–3.50

TABLE II

Results of excretion and clearance studies on abnormal group

Subject	Age	K_E PAH	C PAH	C Inulin	F.F.	Diagnosis
P.C.	43	0.22	115	16	.14	Chronic glomerulo-nephritis.
F.K.	38	0.60	45	8	.50	Hypertension, nephritis.
M.H.	58	0.60	365	47	.13	Membranous glomerulo-nephritis.
J.B.	21	0.24	56	12	.21	Renal failure, recovery phase.
M.B.	42	1.08	494	71	.14	Chronic glomerulo-nephritis.
O.L.	68	1.40	133	81	.60	Possible renal tubule defect.
D.H.	62	0.97	225	48	.21	Essential hypertension.
D.A.	58	0.80	115	69	.60	Collagen disease; brain abcess; gout.
M.P.	64	0.62	171	67	.39	Chronic glomerulo-nephritis; C.N.S. syphilis.
W.H.	65	0.37	872	44	.05	Non-functional right kidney; hydronephrosis; hypertension.
Average	52	0.74	256	45.5	.26	
Std. Dev.	13.7	0.16	81	23.1	.017	
Range	21–65	.22–1.40	45–494	8–81	.05–.60	

Data obtained on the "normal" group compare favorably with that reported by Elliott and Wu,[3] who reported K_E for hippuric acid of 2.70 hr^{-1} in a group of normal subjects.

The three fundamental mechanisms of excretion (glomerular filtration, tubular resorption, and tubular excretion) are said to be altered more or less independently in renal disease. It is known that in glomerulonephritis the glomerular filtration rate will be altered (lessened)

earlier than in hypertension, while function of tubular excretion is reduced first in the latter disease. It is also possible that the renal blood flow may be diminished by causes other than renal disease. The presence or degree of renal impairment is said to be measured by the clearances of PAH, maximal PAH, and inulin.

In Table II the K_E PAH, C_{PAH}, C_{Inulin}, and filtration fraction for various renal diseases are seen. In glomerulonephritis where glomerular filtration is lowered, there is a lowered PAH clearance and K_E PAH. In hypertension these three values are again lowered. K_E PAH appears to be reduced consistently in a somewhat proportionate manner in the various disorders noted.

The fact that PAH is partially metabolized to p-acetylaminohippuric acid (PACA) in man has been established.[4, 5] In these investigations it was found that the excretion constant for PAH does appear to be altered by conjugation of PAH, particularly at low plasma levels (Fig. 3). It was found by Newman[5] that clearances obtained following single injections of p-acetylaminohippurate remained constant.

SUMMARY

1. A method using a single injection of p-aminohippuric acid (PAH) and relating its removal from the body in terms of first-order kinetics as an excretion constant has been described.
2. The procedures are easy to perfom, require only one intravenous injection and several voided urine samples, and can be performed in any laboratory with a colorimeter or spectrophotometer.
3. The functional status of the kidney, in both "normal" and in some renal disorders has been compared by the excretion constant (K_E PAH), and a significant difference is shown between "normals" and cases of renal disease.
4. PAH and conjugated PAH (PACA) in the urine have been studied concomitantly with the differences in the excretion data shown. Determination of "total" PAH in the urine by the method of Newman (1949) can easily be substituted in the analytical procedures.
5. The necessity for further study on "normal" and "abnormal" kidney function as K_E PAH and K_E TOTAL PAH has been indicated.

Acknowledgment

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IN MEMORIAM

We wish to extend our sincere sympathy to the families and friends of:

Elizabeth G. Pape—ALABAMA
Edna Marie Court—ILLINOIS
Reaba M. Smith—INDIANA
Elizabeth W. Tewksbury—INDIANA
Dr. Kano Ikeda—MINNESOTA (Honorary)
Leroy George Murch—NEW JERSEY
James H. Lawrence—NEW MEXICO
Geraldine H. D. Barton—OHIO
Cecilia Hogg—OHIO
John Cleve Waldrop—OKLAHOMA
Mr. J. H. Kieper—OREGON
Jo Ann Marie Viele—Washington

Members of ASMT who died since November (1960)

Egg Yolk as a Medium for Isolation of Brucella*

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Isolation of brucellae from blood or other body tissues of man and animals remains the best evidence for a diagnosis of brucellosis, hence better and more economical media and methods for securing these isolations are continually being sought. Witness, for example, the two phase medium introduced by Castaneda,[2] a selective medium[13] adapted from that of Kuzdas and Morse,[8] the concentration of blood lysates on membrane filters by Braun, *et al.*,[1] and the recent evaluation of brucella media by Morgan.[10]

Embryonated eggs have been shown by several workers to be suitable for growth of brucellae. Goodpasture and Anderson[6] first reported growth of *Brucella abortus* in the yolk sac, and this was later confirmed and elaborated by Gay and Damon[4] who demonstrated that five day old embryonated eggs gave best recovery from small inocula of brucellae. Damon and his associates[3, 5] later made a comparative study of embryonated eggs, guinea pigs, and crystal violet broth for isolation of the three species of *Brucella*. Their results clearly established the superiority of yolk sac inoculation for the recovery of these bacteria from clinical specimens. Other workers[7] have also found the yolk sac particularly suited for the recovery of brucellae.

Use of the yolk sac, though unquestionably superior for isolation of brucellae, is relatively impractical. The expense and difficulty of maintaining a ready supply of fertile eggs and the time required to process specimens by this procedure are not suited to the clinical laboratory. As an alternative to embryonated eggs, we have examined egg yolk as a culture medium for brucellae and found that representative strains of the three commonly encountered species consistently gave growth from small inocula (2-10 organisms). Our evaluation of this medium for the growth of brucellae included studies with blood specimens from rabbits experimentally infected with *B. abortus*, *B. melitensis*, and *B. suis*.

Materials and Methods

All glassware was cleaned in hot sulfuric-nitric acid and thoroughly rinsed in both tap and distilled water. The routine medium for both plates and slants consisted of Albimi's brucella broth supplemented[9] with 100 μGm. per cent nicotinamide, 100 μGm. per cent thiamine hydrochloride and 400 μGm. per cent hemin, and solidified with 2 Gm. per cent agar.

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The egg yolk medium was prepared in the following manner: Twelve uncracked White Leghorn chicken eggs were soaked in 70 per cent ethanol for ten minutes. The eggs were transferred to a sterile bacteriological hood and aseptically broken and manipulated to effect removal of intact yolk. The yolk was homogenized with a magnetic stirrer, dispensed in 3 ml. amounts into 13 x 100 mm. screw-capped tubes, and stored at —20° C. until used.

Eighteen white male rabbits, three to four months old, (six for each species of *Brucella)* were inoculated intraperitoneally with 1 x 10^9 organisms. Cultures for these experimental infections, obtained from the departmental collection, were typical, smooth *B. abortus* 3a-67 (CO_2 independent), *B. melitensis* 4m-64, and *B suis* 3s-91. Suspensions for animal injection were prepared in sterile distilled water from 48 hour (35° C.) slants. Folowing inoculation, blood specimens (10 ml.) were withdrawn from the heart of each rabbit every three to four days, for six weeks, at which time all animals either had died or no longer gave positive cultures.

Each blood specimen was added to 10 ml. of sterile 2 per cent sodium citrate dihydrate in a 25 ml. screw-cap tube. The citrated blood was then concentrated by a modification of the Pickett and Nelson procedure[11] as follows: The plasma was removed, following centrifugation for 30 minutes at 1100 g., and saved for agglutination and bactericidal tests. The cellular elements were lysed with 20 ml. of sterile distilled water and centrifuged for one hour at 1710 g. The supernatant was discarded. The blood cell sediment was mixed with 20 ml. of sterile distilled water and again centrifuged one hour at 1710g. This supernatant was also discarded and the remaining sediment (ca. 2 ml.) was used for inoculation of eggs and media as follows:

(1) a brucella agar primary plate (0.2 ml. sediment) which was incubated two weeks at 35° C. and inspected daily for the presence of colonies.

(2) two tubes of egg yolk medium (0.3 ml. sediment to each) which were incubated four days, then subcultured to brucella agar plates. The secondary, like the primary plate, was incubated two weeks with daily inspection for colonies.

(3) two five-day embryonated eggs. Each yolk sac was injected with 0.3 ml. of sediment by way of a small opening drilled at the air sac end. The openings were then sealed with plastic tape. The embryonated eggs were incubated four days and then 0.1 ml. samples of yolk, aspirated with a syringe, were subcultured onto brucella agar plates. Plates were incubated at 35° C. for two weeks and observed daily for colonies.

All colonies were checked for morphology under 30X magnification with oblique light. Colonies were picked to duplicate tubes of supplemented brucella broth (1.0 ml. amounts) and incubated 48 hours at 35° C. A drop of normal rabbit serum was then added to one tube and a drop of antibrucella serum (agglutinin titer 1:640) to the other. Agglutination was determined after two hours at 56° C. Typical colony morphology and specific agglutination with antibrucella serum constituted the criteria used for positive identification of suspected brucella colonies.

TABLE 1

Number of Isolations Obtained Using Albimi's Brucella Agar, 5 Day Old Chick Embryos, and Egg Yolk as Brucella Blood Culture Media

Organism	Total No. of Cultures	Total No. of Isolations	Isolations from Albimi's Brucella Agar	Isolations from Embryos	Isolations from Egg Yolk
B. abortus	71	26	11	19	25
B. melitensis	54	27	17	22	25
B. suis	61	7	6	6	6

Results and Discussion

The number of *B. abortus*, *B. melitensis*, and *B. suis* isolations obtained with each medium is given in Table 1. The rather low number of recoveries obtained from the infected rabbits, particularly those inoculated with *B. suis*, may be partially explained on the basis of an inherent resistance of rabbits to brucella infection.[12] The egg yolk medium gave an equal or greater number of isolations than either of the other media employed. It is apparent from these figures that no one medium gave 100 per cent of the total number of isolations obtained. In two instances, the embryonated egg was the only medium which yielded colonies of brucellae from blood culture specimens. However, in eight instances the egg yolk medium was singularly responsible for isolation of the organism.

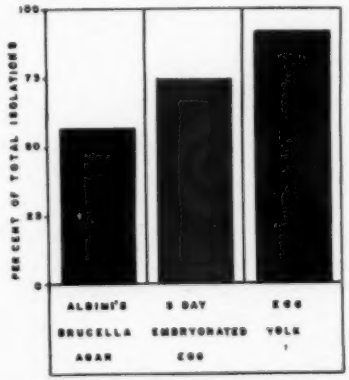

Figure 1. Comparative value of three media used in the isolation of brucellae from the blood of infected rabbits.

The comparative value of the three methods of isolation of brucellae from the blood of infected rabbits is demonstrated in Figure 1. The superiority of egg yolk medium to both five-day embryonated eggs and brucella agar plates is clearly indicated.

In addition to being superior for the isolation of brucellae, egg yolk offers the advantages, in contrast to the five-day old embryonated egg, of being immediately available for use and comparatively inexpensive.

Summary

Five-day embryonated eggs, Albimi's brucella agar, and egg yolk were compared as to their suitability for supporting growth of brucellae from blood of rabbits experimentally infected with these organisms. Tubed egg yolk was found to be a superior medium for the growth of brucellae from small inocula.

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What Can a Medical Technologist Do About The Hazards of Blood Transfusions?*

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In considering how best to present this subject to you, I thought that it should be made as practical as possible. Therefore, I listed several very practical and effective ways that you may avoid the hazards of blood transfusion. These are:

1. Don't be a medical technologist. This is obviously one of the most effective ways of meeting the problem.

2. Work in some place in which blood is never used.

3. Play ignorance. If some doctor asks you to do a Coombs crossmatch or to look for the evidence of sensitization in a patient, be vague; ask him what he means by a Coombs crossmatch. The chances are that not only will he withdraw the request; he will cancel the order for the transfusion and hurriedly leave the laboratory. This may seem a far-fetched solution; I have seen it used (and bragged about) by one laboratory worker on repeated occasions.

4. Ignore these problems; 95 percent of the time nothing happens anyway—the statistics are all in your favor. Just close your eyes and hope that the problems will go away. The late John Elliott of the John Elliott Blood Bank of Dade County said that most of the refinements in the field of blood banking—most of our special efforts—are devoted to the solving of the problems of the troublesome two percent, because the routine operations of a blood transfusion service run smoothly. If he were alive today, I believe that he would certainly raise this figure to five percent.

The shortcomings of the practical suggestions which I have offered are that you *are* medical technologists. Probably many of you are working in transfusion services; therefore you are faced with a real and continuing problem. The fact that you attend meetings which include the presentation of papers such as this one indicates your interest in this field.

Two months to the day on which this paper was read, a young woman of thirty, the mother of three small children, went into a major hospital in a large western city for simple repair surgery. As sometimes happens in such cases, an unexpected loss of blood occurred, and three pints of blood were ordered for her. While she was under anaesthesia, three pints of blood were administered. The only difficulty was that, in the excitement, someone picked up a bottle of blood matched for another patient of a different blood group, and this was one of the three pints of blood which this young mother received. Today, she is dead, a family is shattered, and the lives of four persons are ever changed because of a human error in blood transfusion.

Now this incident did not happen in the days when the science of blood transfusion was young; it did not happen in some far away place

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like Timbuktu; it did not happen in some tiny crossroads hospital. *It happened in a major hospital in a major city in the United States only yesterday.* It *could* happen in your hospital tomorrow or next week—the day after you get back from the annual meeting of the American Society of Medical Technologists, for example.

The whole matter of avoiding the hazards of blood transfusion seems to me to be largely one of attitude. The ingredients of this attitude may be listed as KNOWLEDGE, MENTAL ALERTNESS, METICULOUS ATTENTION TO DETAIL, and a SHARP AND UNRELENTING CONSCIENCE. Let us examine each of these in some detail:

KNOWLEDGE

The only reason for the years of schooling which you have had, for your special training, and even for your registration in medical technology has been to provide you with knowledge and with skills—in other words, with the tools with which to work. This knowledge then becomes a part of your attitude. Because of your knowledge in this field, you have a different attitude about the hazards of blood transfusion than does someone educated along entirely different lines. In fact, the greater your knowledge in this field, the greater is your concern in these matters. Your *attitude* changes.

In connection with this matter of knowledge, try these evaluations on yourself: Do you know where to start to look for the solution to a problem when a blood appears to be incompatible? Do you recognize the importance of controls? Do you know the difference between simulated and true incompatibility? Do you know what are the principles behind these puzzling phenomena? Do you know *why?* It is the knowledge of the *why* which divides you as a trained medical technologist from the mere worker-by-rote.

An excellent illustration of the role of knowledge and the recognition of the *why* of a problem was recently brought to my attention. The patient's physician had requested blood; the routine crossmatch included the indirect Coombs test. Many pints of blood were matched, all with the same results incompatibility by the indirect Coombs test. In other words, the same technic was simply done over and over with no inquiry as to the reason for the difficulty. Finally a technologist demonstrated that the patient's cells were coated with an autoantibody and that this same antibody was present in her serum. So, of course, all of the Coombs tests would be positive. A control run with the first crossmatch on the patient's own cells in her *own* serum would have pinpointed the difficulty immediately.

MENTAL ALERTNESS

This is not the same thing as *knowledge*. You may have a great deal of knowledge and yet be a dangerous person to perform a crossmatch if you are 90 percent asleep, either figuratively or literally. This is a matter of having your wits about you and of an investigative and inquiring mind. More than one potential hemolytic transfusion has been forestalled because the technologist was alert and remembered that the sample of blood labeled Mary Smith today was of a different blood group than the

sample of blood labeled Mary Smith yesterday. My favorite example is one in which blood was matched for a patient, Mary Smith, and given without incident. Two days later, more blood was requested. The technologist went to the same room, the same bed, and asked, "Are you Mary Smith?" The patient nodded pleasantly; the blood was withdrawn and sent to a central blood bank for crossmatching. The blood bank technologist rembered that the blood from Mary Smith two days before was group O; now it was group A. Inquiry revealed that the real Mary Smith had been moved; the patient now occupying the room was a deaf-mute.

Much of blood bank work—especially the drawing and processing of blood—even many day-by-day crossmatching tests—become so routine that there is real danger of our becoming bored. No one has stated this fact better than Allen and his co-workers[1] who wrote with reference to crossmatching for massive transfusion, "This is a laborious job and usually a boring one, since all results are negative in most cases. The magnitude of the procedure, and its tediousness, tend to result in relatively inferior workmanship." When boredom sets in, mental alertness dies, and one of the basic ingredients of the attitude which we are discussing is lost. You then become a dangerous person in this field.

Many of the sources of rare sera or the discovery of new blood factors have been uncovered because the medical technologist was content not merely to find a compatible blood, but was alert to the fact that this off-beat blood had something in it which did not quite fit into the usual pattern. All of the important research is not done in the great medical centers; some of it, particularly in the field of blood groupings, is done almost on a shelf in the corner of a crowded laboratory; certainly some of it has been initiated and carried on because of the mental alertness of medical technologists.

Mental alertness takes still another form—an alertness to the need for service. Unfortunately, some of us have been all too willing to provide only the minimum in service—only the least that we can get by with. We have neglected the special tests which are sometimes required because we are not mentally alert either to their importance or to their necessity. It is easy to say, "We have done it this way for thousands of units of blood and nothing untoward has ever happened." I submit to you the thought that you don't know how much *better* the results could have been had you been alert to the total picture. Because it is a bother to provide service in its highest meaning, it is more comfortable not to be mentally alert, and some of us have abandoned or perhaps never knew this important ingredient of attitude.

METICULOUS ATTENTION TO DETAIL

This, too, is a part of our attitude—indeed, it is a way of life for the blood bank worker. The blood transfusion service is no place for the sloppy or careless worker. Difficulties in typing sometimes occur from dirty glassware, and crossmatch errors result from the neglect of detail which is prone to occur in emergency situations. In some fifteen years of experience of conducting a crossmatching service, every major error which I recall occurred in connection with emergency situations, either

real or alleged. In every one of these, the necessary meticulous attention to detail was bypassed for speed.

Blood bank detail requires that you label everything with which you work. Now some of you have been my students and some of you have heard me talk before, and I can almost hear you groan, "Oh, is she still playing that old broken record?" Yes, I am, and if any group is gracious enough to ask me to talk to it twenty years from now, I shall still say *label, label, label.* Many a patient has been killed because of a clerical error, a mislabeled tube of blood, an unlabeled sample, a wrongly selected bottle of blood.

Blood bank detail also requires that the medical technologist use every device which he knows to control his tests. Most errors in Rh determinations are made because the tests are not controlled; erroneous Coombs tests are reported every day because they are run without controls. *Control, control, control*—this too must be the code of the medical technologist.

Again, in meticulous attention to detail, directions must be read and followed explicitly. If the directions say observe for *two* minutes, don't flip the slide off in ten seconds as I recently saw a laboratory worker do; don't read it in five minutes after you have finished a telephone conversation. *Look* for detail; *follow* detail; *live* detail; this is a part of your attitude in avoiding the hazards of blood transfusion.

A SHARP AND UNRELENTING CONSCIENCE

In this consideration we enter the field of ethics, but this too is a basic aspect of attitude—perhaps the most important. The technologist has to care! All laboratory work is subject to evaluation. In blood bank work alone, the evaluation is the biological test on the patient. After the wrong blood has been given, it is too late to recall even a single drop of it—the mistake has been made, and even the greatest regret and anguish on the part of the technologist cannot reverse the error.

This is the only phase of laboratory work in which the medical technologist really holds the life of the patient in his hands. The hospital, the doctor, and the patient depend upon you. Never get so bored or so tired that you will let yourself bypass the factors of safety. Never become so cynical that you can rest with less than your best. A sharp and unrelenting conscience must be an integral part of your attitude.

Finally, let me say that your efforts to avoid the hazards of blood transfusion will be of no avail unless you gain the confidence and cooperation of your staff physicians—especially the pathologist. It may even be that sometimes a specific blood request is made which you know is not the right one. Only as you have gained the confidence of those with whom you work will you ever be in a position to suggest a safer choice—to discuss the alternate possibilities—to choose wisely when you are left alone and are faced with an emergency.

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The Growing Problem of Leptospirosis*

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"Leptospirosis is not a new disease. A disease of man characterized by fever, jaundice, and hemorrhage was observed over a century ago. In 1886, Weil, for whom the disease was named, described differentiating characteristics. Since the discovery by Inada and his associates in 1916 and almost simultaneously by Uhlenhuth and Fromme of the casual organism of Weil's disease, our knowledge of the infections caused by these organisms has considerably increased. In 1918, Noguchi coined the name Leptospira for the offending spirochaete. A similar disease of dogs was described by Hoger in 1850 which became epidemic in Germany in 1898, where it was called Stuttgart disease."[10]

Although research workers had accumulated much information regarding this pathogen, the infection did not attract much attention until it was shown to have specific etiology and to exist in rodents, pets, and domestic animals in addition to man. The first detailed report conclusively establishing existence of canine leptospirosis in the United States was subsequently published by Meyer et al, in 1939, approximately 20 years ago.[10]

The incidence of leptospirosis in the United States is higher than reports indicate, and the disease should be suspected in all patients with undiagnosed febrile illness. Investigators have observed that 5% or more of fevers of unknown origin in hospitals may be attributed to leptospiral infections.[7] In medical as well as in veterinary practice, this is a field of research in which close cooperation between clinician, laboratory and epidemiologist has been exceedingly fruitful. Once attention was focused on the occurrence of leptospirosis in a certain area, further cases were nearly always detected when a thorough search was made by screening the patient's surroundings. Moreover, in many instances mild and even inapparent cases were then found which would have been overlooked by clinical investigations alone. As the knowledge of the etiologic, clinical and epidemiologic aspects of leptospirosis grew, combined with laboratory studies,

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it soon became apparent that in many, if not most, clinical symptoms alone were of insufficient diagnostic value to differentiate the different species of Leptospirae. As a result, cultural and serologic procedures became more and more important as a diagnostic aid.

Cases of leptospirosis have now been detected in nearly every country in the world. Nevertheless, our knowledge of the evaluation and interpretation of the various laboratory procedures for the investigation of leptospiral diseases in men and animals is far from complete. The number of antigenically different strains of leptospirae isolated in different parts of the world is continually increasing, and differences in results obtained by the so-called established laboratory methods point up the need for international cooperation in comparing techniques for leptospiral research. This cooperation has been inaugurated by the members of the Leptospira Subcommittee of the Nomenclature Committee of the International Association of Microbiologists.

Epidemiology

Rodents, swine, cattle and dogs may become naturally infected with leptospira in addition to other wild and domestic animals. In a study of 2,501 wild animals in southwestern Georgia in 1959, 10% were positive for leptospirae (by culture); and 18% by complement fixation. The cottontail rabbit and spotted skunk were recorded as new hosts for leptospirae. In 1937, Meyer and associates stated that "Since it is now proven that, in certain areas of California, as many as 34% of the dog population is subclinically infected with L. canicola, urinary shedders are doubtless quite common."[10]

The greatest danger of infection in man is from contact with stagnant water to which wild or domesticated animals have access, but infection may result from direct contact with the urine of infected animals as well.

In a serological survey of 659 dogs for leptospira using the capillary tube method and L. canicola, L. icterohemorrhagiae, and L. pomona antigens, from the Chicago area, 39.4% of the males and 30.7% of the females were positive.[9] In the south, 32.3% of the males and 23.2% of the females were positive. (It is presumed that the reason for the higher incidence in male dogs is their habit of sniffing the genitalia of other dogs).

It is known that leptospira are capable of surviving and remaining virulent for a period of three weeks in stagnant water that has a reaction which approaches neutrality. The organisms usually die at a pH less than 6.7.

The portals of entry are the skin and mucous membranes. The organism does not have the ability to penetrate unbroken skin. The greatest frequency is in rice field workers, cane cutters, swineherds, cattle farmers, slaughterhouse workers, miners, sewer workers, veterinarians and soldiers.[6]

In addition to being a public health menace to man, leptospirosis caused losses due to abortion in cattle and swine, and retarding influences on growth and milk production in the United States is estimated to exceed $100,000,000 annually.

Manifestations

Leptospirosis is akin to that "Great Imitator"—Syphillis, in that it may manifest itself in so many different ways. It may be presented to the clinician as a gastro-intestinal disturbance, an undiagnosed febrile illness, headache and myalgia, meningitis, jaundice, or as a condition similar to many other central nervous system infections, and frequently mimicks poliomyelitis or tuberculous meningitis.

The diagnosis of leptospirosis should be considered in any patient with an acute illness, characterized by headache, myalgia, fever, chills, and conjunctival suffusion, and in any patient in whom aseptic meningitis develops following an acute febrile illness of five to seven days' duration.

In a report in the American Journal of Medicine, July, 1959, the author cited conjunctival suffusion and pleocytosis as diagnostic clues. Twelve case histories were presented, in which the symptoms varied considerably. Leptospirosis usually commences abruptly after an incubation period of three to nineteen days, with fever, myalgia, prostration, conjunctival injection, anorexia, abdominal pain, nausea and vomiting. Fever lasts about a week. All patients have headache, and about half also have retro-bulbar pain. The headache is usually frontal in location (sometimes bitemporal or occipital) and is described as intense and unremitting (rarely throbbing) in character. It tends to persist throughout the first stage. About one-fourth of patients have a rash consisting of small macules and papules on the trunk and extremities, which appears after the fourth day. The rash at times may assume a petechial character. Cough is common and the chest film may reveal pneumonitis.

The incidence of hemorrhage depends on the severity of the disease. The lung, gastro-intestinal tract, conjuctiva, nose and skin are the commonest sites. Almost half of the patients have lymphadenopthy, but splenomegaly is rare. Nuchal rigidity is frequent. With extensive infection, jaundice or renal insufficiency occurs as the fever is subsiding. In the patients who develop conjunctivitis, usually only the bulbar conjunctivas are suffused, giving the eyes a bright pink appearance similar to that which occurs after the eyes have been exposed to fresh water during swimming.

Leptospirosis appears to be a biphasic illness. Initially, there is a five to seven day period of fever, chills, headache, myalgia, conjunctivitis, and gastro-intestinal symptoms. Since leptospires can be recovered from the blood and cerebrospinal fluid in a high percentage of cases, it has been designated as the "septicemic" stage.

The second stage begins as defervesence and symptomatic improvement occurs on the sixth or seventh day. Shortly following the end of the first stage, there is usually a secondary rise in temperature and development of meningitis, either asymptomatic or clinical. It has been demonstrated repeatedly that the blood and cerebrospinal fluid are sterile during this stage, organisms rarely having been recovered after the seventh or eighth day.[3] However, some instances are cited in the literature in which leptospirae were found in the blood as late as the tenth or eleventh day (Hubener-Reiter, 1916; Taylor-Goule, 1931; Senekjie, 1944; and Willcox, 1944). In Kotter's case (1939), a serologically aberrant strain was reported recovered from a blood culture

on the fifteenth day of the illness in a patient with leptospirosis febrilis in Indonesia; the question arose whether a relapse might not have caused a second invasion of the blood.[18] These instances are regarded critically by most authors as to the correctness of the exact date of the illness or the validity of the dark field diagnosis.

During the second stage, antibodies appear in the blood in rapidly rising titer. In addition to fever and meningitis, several infrequent manifestations such as iridocyclitis and various neurological lesions may develop. Several of the authors reviewed in this study regard these second stage manifestations as primarily due to hypersensitivity, and it has been proposed that this phase be designated as the "immune" stage, since the previously suggested terms "icteric" stage and "toxic" stage are inappropriate because of the low incidence in leptospirosis in general and because there is little evidence that toxins are important in the production of the principal manifestations. Gsell stressed the biphasic character of leptospirosis in his monograph. In addition, since the blood and spinal fluid are sterile in the second stage, the manifestations must have a different pathogenesis than those of the first stage The fever of the second stage occurs at a time when antibody titers are rising rapidly and leptospires are being destroyed, thus suggesting the possibility that it is on an allergic basis.

Morphology and Staining Characteristics

The morphology of the microoganism as seen in dark field or in stained preparations was described very accurately in the early period of leptospiral research (Noguchi, 1918; Zuelzer, 1918; and Pettit, 1928). Leptospirae are slender thread-like organisms without flagella. They possess very minute spiral coils having an amplitude of about 0.5 micra. Electron-microscopic measurement suggest that their thickness may be less than 0.1 micron (Babudieri, 1948). Their length is very variable and may be from 6 to 12 micra, but sometimes, particularly in old cultures, very long specimens of 30-40 micra can be detected.

When living leptospirae in a fluid medium are examined with lower magnifications in dark field illustration, the primary spiral coils are scarcely visible; the impression usually obtained is that of a row of small dots or a string of fine beads. Their movement is very peculiar; the middle part is held rigid while one or both ends, bent like a coat hanger, are seen to make quick twists and whiplike darts so that rotations along a longitudinal axis occur.[18]

Various aspects of the exact internal structure of the organism remain in dispute; for example, the presence or absence of a rigid central filament, the exact structure of the terminal part of the leptospiral body, etc.

Although this morphological appearance is typical of the genus Leptospira, species diagnosis cannot be made on this aspect alone because of size variation and in sufficient differentiation between saprophytic and pathogenic leptospirae.

The relation of the saprophytic leptospirae to the pathogenic leptospirae is uncertain. Uhlenhuth and Zuelzer[2] have reported observations

which suggested that the water leptospira (L. biflexa) are avirulent forms of L. icterohemorrhagiae whose virulence may be restored by animal passage. These findings have not been confirmed by other works. Leptospirae are Gram-negative microorganisms; however, they stain very poorly with the usual bacterial stains. The inability of these organisms to stain with ordinary bacteriological stains is supposedly due to their lack of nucleoprotein, but this has not been demonstrated conclusively by analytical chemical methods. Generally, better results are obtained by using concentrated Geimsa solutions. Viktoria blue (No. 4 R, concentrated) has been used by German workers and Burri's method using a mixture of the material and India ink brings out the typical morphology clearly. For staining in paraffin sections of tissues, Levaditi's silver impregnation method is recommended.

Type Stains of Leptospira

Because leptospires cannot be satisfactorily studied by the methods used for the true bacteria or even for other spirochetes, it has proved difficult to apply the species concept to this important group. The differentiation of strains of leptospires is however of epidemiological importance as well as of academic interest. Various schemes of differentiation have been used, but at present the most satisfactory method is based on the antigenic constitution of the leptospires, which can be determined by agglutination-lysis and cross-absorption tests. Complement-fixation tests have not yet been developed as a means of typing, neither has any system of cultural or biochemical means been devised which could be used as a basis for differentiation.

Wolff and Broom compared the serological characters of a number of strains of leptospires whose origins and subsequent histories could be fully authenticated. These workers assembled into "serogroups" (serological types) serotypes which showed marked similarities in their serological reactions. These serological groupings quite probably have some relation to the differentiation of the organisms into species, but it is considered that knowledge of the bionomics of leptospires is not yet sufficiently far advanced to allow a decision to be taken regarding the criteria on which the subdivision of the genus should ultimately be made. Hence, for the time being, it is proposed that the term "serotype" should be adopted to designate the basic taxon of a serological classification based on the agglutinogens of the leptospires.

The number of antigenically different strains isolated from cases of human and animal leptospiroses is continually increasing. This presents a complex and confusing picture which will only be clarified by further investigation and international cooperation. The author will later in this paper present in table form the type strains of leptospirae as presented in Bergey's *Manual of Determinative Bacteriology*,[1] but in the body of the paper will confine her remarks to the well-established serologic entities, since our knowledge of the position of other strains isolated from human and animal sources is still very incomplete.

L. icterohaemorrhagiae, isolated by Inada-Ido, 1915, is the causative

organism in classical Weil's disease. It is world-wide in distribution. The principal animal host is R. rattus decumanus (wild rat), but occasionally a great number of other rodents and dogs have been found to be naturally infected. High cross reactions may be encountered with L. canicola. This serotype was responsible for one case of twelve leptospiroses reported in the American Journal of Medicine, July, 1959.

L. canicola, the causative organism in canicola fever, or leptospirosis canicola, is worldwide in distribution and has as its principal host, dogs. L. canicola was first isolated by Klarenbeek and Schuffner in 1933. The leptospiras were differentiated from L. icterohaemorrhagiae by serologic methods. It has been said that L. icterohaemorrhagiae can be differentiated from other blood spirochaetes by its insolubility in a 10% solution of saponin. No data were found on the reaction of the 10% saponin solution on L. canicola. The first cases of this disease in human beings were reported by Roos, Walch-Sorgdrager and Schuffner in 1937, when they reported 11 cases from Holland. The first case of the disease in human beings in the United States was reported in 1938 by Meyer, Eddie and Stewart-Anderson from San Francisco. This disease is fairly rare, there being only 31 cases on record from the entire world, and only 6 from the United States. Cases have been reported from Holland, Austria, Denmark, and China; and from Washington, D. C., San Francisco, and New Orleans in the U.S. Undoubtedly, all cases seen have not been reported, and it is quite possible that a few of the cases were missed in the survey of the literature. Certainly, more cases will be found as the medical profession becomes more acutely aware of its presence and consequently looks for it more frequently, as was the case with Weil's disease. At the beginning, the clinical picture of this disease is strikingly similar to that of Weil's disease, but generally speaking, canicola fever tends to be milder than the illness caused by L. icterohaemorrhagiae.

L. grippotyphosa was first isolated by Tarassoff, 1928, and is the causative species of swamp fever of eastern Europe, also known as mudfever. The principal animal host is Microtus arvalis (field mouse) and other voles. The distribution is principally in eastern Europe, although in the survey of twelve cases of leptospirosis appearing in the July 1959 issue of the American Journal of Medicine, this serotype was incriminated in three of the twelve cases, and positively identified in one case as the sole organism; the author laid claim to the first (reported) case in the United States.[3]

L. hebdomadis was isolated by Ido, Ito, and Wani in 1918. This is the causative serotype in Japanese seven-day fever or nanukayami. It is found in Japan, Formosa, Indo-China, and Indonesia. The principal animal host is Microtus montebelloi (field mouse). A common sequel to this infection is opacification of the vitreous humor.

L. sejroe, isolated by Borg-Peterson, 1938, causes an infection resembling mudfever found in cases of jaundice particularly in Denmark. The principal animal hosts are voles and field mice.

L. autumnalis, isolated by Koshina et al., 1925, causes autumnal fever.

or akiyami (hasami fever) of Japan. This species has also been incriminated in several cases in the United States.

L. pomona (Clayton-Celento-Derrick, 1937) is the causative organism in swineherd's disease. This serotype may also be the cause of bovine leptospirosis in cattle. The principal animal host is the pig. Distribution is probably world-wide. This strain is found frequently in human cases of leptospirosis in the United States.

L. pyrogenes (Vervoort, 1923) is the causative species of Sumatra and Java jaundice or spirochaetosis (leptospirosis) febrilis. The principal animal host is unknown.

A list of the recognized serotypes taken from Bergey's Manual follows:

List of Recognized Serotypes

Serogroup	Serotype	Type Strain
Icterohaemorrhagiae	icterohaemorrhagiae (AB)	M 20
	icterohaemorrhagiae (A)	RGA
	naam	Naam
	Mankarso	Mankarso
Javanica	javanica	Veldrat, Batavia 46
	poi	Poi
	sarmin	Sarmin
	Schuffneri	Vleermuis 90 C
	canicola	Hond Utrecht IV
	benjamin	Benjamin
	ballum	Mus 127
Pyrogenes	pyrogenes	Salinem
	australis B	Zanoni (C. 14)
	cynopteri	3522 C
	sentot	Sentot
Autumnalis	autumnalis (AB)	Akiyami A
	autumnalis (A)	Rachmat
	bankinang	Banginang I
	dyasiman	Djasiman
Australis A	australis A	Ballico
	muenchen	Muenchen 90 C
	pomona	Pomon
	grippotyphosa	Moscow V
Hebdomadis	hebdomadis	Hebdomadis
	medanensis	HC
	wolffii	3705
	hardjo	Hardjoprajitno
	sejroe	M 84
	sakoebing	Mus 24
Bataviae	bataviae	van Tienen
	paidjan	Paidjan
	semarang	R.S. 173
	adaman A	CH 11
	hyos, syn. mitis Johnson	Mitis Johnson

Diagnostic Procedures and Laboratory Findings

In order to provide a basis for the choice of diagnostic method, the detailed discussion of these methods is prefaced by an outline of the stages of the disease and the procedures to apply in each stage:[8]

I. Acute, febrile, septicemic stage—3rd to 7th day.
  a. Demonstration of organism
    (1) Circulating blood
      (a) Culture and animal inoculation best.
      (b) Examination of dark-field and stained preparations less satisfactory.
    (2) Bone marrow
      (a) Culture and animal inoculation possibly positive.
      (b) Direct examinations rarely positive.
  b. Detection of antibodies—rarely positive.

II. Hepatic or icteric stage—7th to 10th day.
  a. Demonstration of organisms
    (1) Blood—less likely to succeed than in first stage.
    (2) Urine—occasionally animal inoculation and culture positive; other procedures useless.
    (3) Cerebrospinal fluid—occasionally animal inoculation and culture positive; other procedures valueless.
    (4) Bone marrow—culture and animal inoculation may be positive; other procedures are rarely positive.
  b. Detection of antibodies
    (1) Blood—often positive findings.
    (2) Urine—less often positive.

III. Convalescent Stage—often prolonged.
  a. Demonstration of organism
    (1) Blood—rarely positive by any method.
    (2) Urine—usually positive by animal inoculation and culture.
    (3) Cerebrospinal fluid—occasionally positive by animal inoculation and culture.
    (4) Bone marrow—data insufficient for evaluation of the methods for demonstration of organism.
  b. Detection of antibodies
    (1) Blood—usually positive findings.
    (2) Urine—less often positive.

IV. Postmortem—organisms may be demonstrated by culture, animal inoculation, or silver impregnation of liver, kidney and adrenal tissue.

Blood

During leptospiremia, organisms may be detected by dark-field examination of a drop of blood plasma under a cover glass with a dry power objective. Unfortunately, this method is far from infallible. Care should be taken to distinguish between "pseudospirochaetes" which have about the same dimensions as leptospirae, but are probably protoplasmatic threads derived from blood platelets and erythrocytes. They never show the typical active movements of true leptospirae with rigid-

ity of their central parts, but may bend and stretch in all parts of their longitudinal axis as a result of the fluid stream, or perhaps as a result of Brownian movement of particles. The chance of finding leptospirae in the blood of proved cases of leptospirosis is of the order of 8%.

Routine laboratory observations disclose little, since there may be no change in the white blood cell count, or a moderate leukocytosis. In one survey of cases, a range of white blood cell counts from 4,400 to 13,400/ cu. mm. was disclosed in the anicteric patients, while the jaundiced cases disclosed leukocytosis ranging in severity up to 35,000/ cu. mm. Neutrophilia of 80 to 90% is common, even when the leukocyte count is normal. Liver function tests, including bilirubin, alkaline phosphatase, cephalin flocculation, and albumin and globulin may be abnormal even without icterus. Elevated sedimentation rate is a fairly constant finding.

Urine

Examination of the patient's urine is indicated after the first week of the disease. In the later stages, discharge of leptospirae in the urine is often very irregular and it is quite possible to have several negative results followed by an occasional demonstration of leptospirae. Leptospirae are very sensitive to acid; therefore it is recommended that an alkaline diet be given to the patient for several days before the examination to increase the chance of a positive finding.

Direct cultural tests of urine samples seldom yield positive results, owing to bacterial contamination of the urine. Good results may be obtained from the injection of guinea pigs intraperitoneally. The strain may best be isolated by inoculating media with blood obtained by cardiac puncture.

Protein, casts, and cells may appear in the urine, and oliguria and anuria may occur. In icteric cases, bile may be present in the urine.

Cerebrospinal Fluid

During septicemia, leptospirae may be present in the cerebrospinal fluid and Fontana's stain is best used in the search for them. They appear there some days later than in the blood and may be detected by direct dark-field observation of a drop of the fluid or by animal inoculation and cultural tests.

During the later stages of the disease, antibodies can also be demonstrated, but the titer is much lower than in the blood serum. Cerebrospinal fluid findings in leptospirosis are not diagnostic. The findings in leptospiral meningitis vary from a normal fluid early in the course of the disease to mild pleocytosis and a mild increase in protein later. Sugar and chloride levels usually remained within normal limits, although there is a temporary depression of spinal fluid sugar in some patients. The frequency of aseptic meningitis is not known. Spinal fluid usually shows 40 to 500 cells, predominantly mononuclear. Spinal fluid alterations are of no apparent prognostic value. In some instances the meningeal manifestations may appear as the most dominant feature of the disease, either prior to the onset of jaundice or among the patients without jaundice. These phenomena call for a refined diagnostic accuracy

and an increased level of suspicion of the disease. The examination of the spinal fluid in the light of the clinical picture may aid the establishment of the diagnosis of the disease through isolation of the leptospirae organisms. However, a bilirubin-tinged spinal fluid is of limited diagnostic value, since a clear fluid is the rule, even in the presence of high serum bilirubin levels.

The pathogenesis of xanthochromia of the spinal fluid in classical Weil's disease remains unexplained. It may be governed by an increased capillary and meningeal permeability from either the direct effect of the leptospira organisms or the complicating renal failure. It is emphasized that the anicteric form of leptospirosis is at times indistinguishable from other acute infectious diseases, mainly severe influenza without prominent signs of an acute respiratory infection. The final diagnosis must depend on *laboratory confirmation.*

Cultural Procedures and Media

The leptospiras are the most readily culturable of the spirochaetes. They may be grown in a liquid medium consisting of 1% peptone in phosphate buffered Ringer solution to which sterile rabbit serum is added in a final dilution of 1:40, or in a semi-solid medium consisting of meat extract agar enriched by the addition of sterile serum.

The first attempts to culture leptospira employed an anaerobic medium originally devised by Noguchi for the pure culture of Borrelia recurrentis (1916). Some years later, Vervoort (1923) developed a medium through extensive studies of leptospiral infection in Indonesia. Vervoort's studies showed that:

a. Small amounts of protein, preferably peptone "Witte" were necessary.

b. Addition of inactivated rabbit serum or whole blood promoted the growth considerably, a fact already known.

c. The reaction of the final solution was of the utmost importance, as leptospirae proved to be very sensitive to acid or strong alkaline reactions. A pH of 7.2 to 7.4 gave the best results.

In later years, Vervoort's work was thoroughly evaluated by Schuffner et al., in Amsterdam and a few modifications made which gave still better and more constant results.

The stock medium consisted of a solution of 0.1% peptone (Witte) and .05% sodium chloride standardized to a constant pH by adding a certain amount of Sorensen's buffer fluid, pH 7.2. To this basic medium was added either 10% inactivated rabbit serum, a few drops of whole rabbit blood or hemoglobin (laked rabbit blood). It was found that the use of this modified Vervoort's medium resulted in excellent growth of all kinds of leptospira and it is at present widely used in laboratories in various countries.

Basically similar but different in some details are the media developed by Korthof (1928) and Fletcher (1928), of which also variations have been published.

The Public Health Laboratory of the Communicable Disease Center in 1955 published a pamphlet giving a simplified procedure for the prep-

aration of Schuffner's medium which is not quite so tedious to prepare as Schuffner's, but which has given satisfactory results. The medium is prepared from two stock solutions, eliminating the repeated boiling and the addition of phosphate salts at different intervals. An apparatus which facilitates the addition of sterile rabbit serum is also described. The medium has not been tested as an isolation medium.

By and large, however, most authors still refer to Schuffner's medium as the medium of choice since it is free of the agar particles contained in Fletcher's medium, and is ideal for growing organisms which can be used without further treatment as antigen in the agglutination-lysis test. In Schuffner's medium, chemically pure salts and fresh distilled water (preferably double glass-distilled) or rain water must be used to make the necessary buffer solutions while the reaction of the different solutions should be carefully controlled. Failure to do this may be a cause of poor growth. The salt content of the medium is critical; too high a concentration will kill the spirochetes. Growth proceeds only under aerobic conditions, the organisms usually growing in the narrow zone immediately beneath the surface of the medium. The heavier growth obtained in agitated Erlenmeyer flasks containing glass beads and in flat-sided bottles laid on their side is probably due to increased surface area and oxygenation of the medium.

The source of peptone is very important. In most peptone "Witte" is recommended, a German brand which is very suitable for promoting good growth. The rabbit or horse serum makes the medium a rich one for bacterial growth. It is, therefore, essential to insure the sterility of the medium since bacterial contaminants are usually fatal to the spirochetes within 24 hours.

Fletcher's medium is prepared by heating a 12% solution of rabbit serum in distilled water to 50° C., adding 6 ml. of 2½% melted nutrient agar to every 100 ml. of serum-water mixture, adjusting the pH to 7.4, tubing in 3 to 5 ml. quantities and sterilizing at 56° C. for 1 hour on two successive days.

Generally, no matter which medium is used, microscopic examination is necessary to determine whether organisms are present.

In recent years, several new semi-synthetic curture media have been devised from studies of the metabolism and nutritional requirements of pathogenic leptospira. It was found that the addition of serum (preferably rabbit serum) to the medium is indispensable for optimum growth as the rabbit albumin could not be replaced by various amino acids. For the growth of L. canicola, thiamine was found to be the only essential vitamin; a number of other pathogenic leptospirae grow well in a medium containing only salts, thiamine, asparagine and rabbit serum albumin. Small amounts of hemoglobin, yeast extract, and hydrolyzed casein, added to the basic medium, were observed to promote the growth.

In a personal communication from the Veterinary Diagnostic Laboratory, Auburn, Alabama, Dr. Robberts added several practical points pertaining to the growth of leptospiral cultures. This laboratory carries the stock cultures on Chang's medium, a semi-solid medium, and it is necessary to transfer two or three times in Schuffner's medium before

it is suitable for an antigen (because of the small agar particles, etc. interferring with the interpretation of the test). They found also that growth is much slower in Chang's than in Schuffner's, and that it is not necessary to subculture but once a month in order to maintain viability. Their cultures are incubated at 25° C., since growth seems to be more uniform at this temperature than at 37° C., and they do not get as many clumps or microcolonies.

Animal Inoculation

In the beginning of leptospira research, it was found that young guinea pigs were very susceptible to an infection with L. icterohemorrhagiae. In typical cases, after intraperitoneal inoculation of infectious material, leptospira may be found in the peritoneal fluid as early as the third day after inoculation and for some days afterwards. During the same period the blood becomes positive. Contrary to what is seen in humans, death in guinea pigs occurs mainly during the septicemic period. In later years, when the many different type strains of leptospira were recognized, it became apparent that not all strains were pathogenic to guinea pigs. Nevertheless, during the first stage of a suspected leptospirosis, inoculation of blood into guinea pigs is always indicated and may be of much help in diagnosis or in obtaining a pure culture.

Animal inoculations are indicated both in the septicemic period (blood) and at later stages (freshly voided urine). Young guinea pigs, weighing less than 150 grams, preferably white to facilitate the observation of jaundice of the skin, young golden hamsters, and species of deer mice are the animals of choice for the diagnosis of leptospirosis. Certain strains of young white mice are also susceptible.

Typical experimental leptospirosis lasts 5 to 12 days and terminates in death. Conspicuous symptoms during life are fever, jaundice, and conjunctival congestion. Fever usually commences the day after infection, rises to a peak in several days, and descends just before death. Jaundice is first apparent when the temperature begins to fall. It then continues to increase until death. Loss of weight, weakness, drowsiness, inanition, and anorexia are commonly present. At necropsy, the findings are emaciation, intense icterus of the skin and all internal surfaces, multiple petechial hemorrhages in skin, subcutum, muscles, mucous membranes and viscera. The hemorrhages in the lung give this organ the appearance of a butterfly, hence the term "butterfly lung." At postmortem, the organisms are most numerous in the liver, kidney and adrenal tissue.

Further studies are urgently needed at this time in the diagnosis and culture of leptospira.

Serologic Procedures

Studies of leptospirosis in man and animals have been handicapped by the lack of a simple serologic test and of stable leptospiral antigens. Several tests have been offered in the past, all with varying drawbacks.

The microscopic agglutination-lysis test is the most reliable and sensitive of the agglutination procedures, but has the disadvantage of requiring dark-field illumination and is relatively time-consuming to perform. In addition, the laboratory must have a constant supply of

living antigens which are unstable and of rather brief duration. The agglutination-lysis test of Schuffner and Mochtar is the most satisfactory.

Schuffner recommends a formalinized antigen made from a 5 to 7 day culture of leptospira on Schuffner's medium. Control antisera is produced in rabbits by injections in gradually increasing doses of a virulent culture. Agglutination tubes or porcelain plates may be used as receptacles for the antigen-antibody mixtures. Sera are inactivated. Incubation is for two hours at 37° C. for live antigen, and four hours at this temperature for killed antigen, preferably in a water bath. Known positive and negative sera should be included in each series of tests. Usually 0.15 ml. of antigen are added to 0.15 ml. of serum. A drop of the antigen-antibody mixture is examined by dark-field microscope. In a typical positive test, living antigen is agglutinated in lower dilutions and lysed in higher dilutions, while killed organisms usually are clumped in the highest dilutions compatible with the strength of the serum.

When performing the test with living microorganisms two phenomena, agglutination and lysis, occur simultaneously in the higher dilutions of the serum-antigen mixture. Moreover, the lysis differs from the well-known bacteriolysis in that it takes place without the presence of complement (the amount of complement present in the dilution of the serum in which lysis may be present is negligible), and the same reaction is observed when performed with inactivated serum. Further, lysis is nearly always inhibited in the lower serum dilutions as is also often observed with agglutination, a phenomenon resembling the prozone effect in bacterial agglutination.

Pot, Smith, and Tulloch have described macroscopic tests using formolized and live organisms. As the antigens are difficult to prepare and the tests difficult to read, for routine use they have little advantage over the microscopic test. Brown has reported a rapid presumptive test employing a formolized culture in which the serum-antigen mixtures are rocked on a slide and observed with a hand lens against a dark background. Recently Fineman and Joyner have modified Brown's test. These procedures are not used extensively as diagnostic tools and are considered less sensitive than the microscopic agglutination-lysis test.

Other workers have employed the principle of complement fixation. Gaehtgens centrifuged leptospirae from cultures and resuspended them in 0.3% phenolized saline. This method compared favorably with the microscopic agglutination-lysis test, except in 7%, in which the agglutination-lysis test was positive, but the complement fixation test negative. Randall et al., employed complement-fixing antigens from strains of L. canicola and L. icterohemorrhagiae by sonic vibration of saline-washed leptospirae. There was close agreement by this method, but no distinction between the two antigens. York used formalin-killed antigens for complement fixation from L. pomona cultivated in fertile hen's eggs.

Complement fixation tests are easier to carry out and to read, but have so far been less specific and cross reactions with related strains are more frequent. Moreover, positive reactions usually occurred at a later stage of the disease than with the agglutination-lysis technique. In addition, the complement fixation test requires repeated standardiza-

tion of reagents and hence, is not suitable for routine use in the average laboratory.

Stoenner attempted to eliminate the disadvantages of the previously described leptospira antigens. He developed a macroscopic capillary tube test and later adapted this procedure to the macroscopic plate procedure with excellent results. Hoag, Gochenour and Yager modified Stoenner's antigen by adsorption onto cholesterol platelets and claimed thereby to increase its sensitivity.

The adhesion or thrombocytobarin reaction is conducted by adding a mixture of spirochetes and specific serum to a suspension of blood platelets or bacteria, incubating for 20 minutes at 30° C., and observing the preparation by dark-field. A positive reaction is indicated by the platelets or bacteria adhering to the spirochetes forming small clumps. Living motile spirochetes and fresh complement are required. By this test it is possible to distinguish between two closely allied forms like L. icterohemorrhagiae and L. hebdomadis. The usefulness of this method for diagnosis of the disease remains to be determined. There is one report in the literature on the use of a skin reaction for the diagnosis of leptospirosis, but it has never been confirmed.

More recently, Galton, Powers, Hall and Cornell have described a stable, killed leptospiral macroscopic slide antigen which eliminates the disadvantages of former antigens and possesses a sensitivity equivalent to that of the microscopic test. An antigen prepared according to their procedure and recommended for use in the rapid macroscopic slide agglutination test for the serodiagnosis of leptospirosis in humans and animals was used by the author in her survey of the hospital population and dogs of West Jefferson County. These antigens are stable for at least 18 months when properly handled and refrigerated. Excellent results were obtained using positive dog sera.

Prognosis and Treatment

The case fatality is variable; it has varied from 4.6 to 32% in Japan and has been about 10% in the Dutch cases and as high as 25% in Scotland. At autopsy the leptospirae are found in almost all the organs and tissues in those dying during the initial febrile stage, but if death occurs in the second week or later, they are rarely found elsewhere than in the kidneys.

Death is almost always attributable to renal failure with hepatic insufficiency but sometimes is ascribable to peripheral vascular collapse or hemorrhage. Recovery from the disease is accompanied by the development of a solid immunity, and a specific lysin is present in the blood which persists in detectable amounts for several years. Agglutinins appear in the convalescent stage, sometimes to very high titer, but the relation of demonstrable antibody and effective immunity is far from clear. Prophylactic inoculation has given encouraging results.

The chemotherapy of human leptospirosis has not been successful. Penicillin, streptomycin, aureomycin, chloramphenicol and terramycin have all been tested and found ineffective. Although the value of penicillin and tetracyclines in therapy is doubtful, the administration of four to six million units of penicillin a day is probably advisable when infection is extreme. Renal failure demands meticulous fluid and electro-

lyte management and, in some instances, dialysis with the artificial kidney.

The treatment of leptospirosis with arsenicals has been unsatisfactory. Better results have been reported with soluble bismuth preparations. There is considerable dispute as to whether the bismuth kills the spirochetes, slows them up, or whether it merely speeds up the normal body defense mechanism.

It can be said that whatever specific treatment is used against leptospirosis should be given early. At present, most of the diagnoses are made serologically, and consequently late in the course of the disease. It is unlikely that any specific therapy will be of value until the presence of the disease is appreciated sufficiently by the medical profession, so that a diagnosis can be made early.

Survey of Human and Animal Hospital Population of West Jefferson County

Using the macroscopic slide leptospira antigen perfected by Galton et al., the author has performed rapid slide agglutination tests on the serum of more than 250 patients in Lloyd Noland Hospital for the detection of leptospirae agglutinins. To date, no positive reaction has been detected.

In the tests on dogs, however, some 25 dogs admitted to Dr. K. U. Jones' Animal Hospital in this area have given positive reactions in high titer to formalized suspensions of individual and pooled Leptospira serotypes. The two serotypes found in this survey were L. icterohemorrhagiae and L. canicola.

Summary

A review of the literature concerning leptospirosis in general and in particular is presented. Results of a survey of humans and dogs for leptospiral antibodies conducted by the author are presented.

There are a number of problems which must be solved in the diagnosis, culture, and treatment of leptospirosis.

A medium which will enhance the growth and speed it up more than those now available is desperately needed. Possibly a refinement of the chick embryo method of cultivation of leptospira will facilitate diagnosis. The development of differential media to reduce contamination and also to distinguish between the many serotypes would be most helpful.

It is the opinion of the author that the two serotypes found to be the causative species of leptospirosis in dogs in this area is probably due to the occurrence of cross reactions between L. icterohemorrhagiae and L. canicola, and that the offending organism is actually L. canicola.

Through the improvement of diagnostic methods or more intelligent use of those procedures we now have, it is quite likely that leptospirosis in man and animals will be found to be more common in the United States and in the world than is now appreciated. Already the animal reservoir appears to be widening; in time it may be found to be a public health problem in the United States and elsewhere.

Finally, it is the opinion of the author that cases of leptospirosis in man do exist more commonly in this area than is generally believed.

The organism has been proven present in animals, both domesticated and wild, and certainly must find its way into man also.

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Scientists Should Be Leaders*

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There is great need for better leaders in organizations, civic activities, associations such as parent teacher groups, and in professional groups. Employers are constantly searching for supervisors who are real leaders, and universities desire teachers who are an influence for good.

Some of the qualifications for leadership are willingness to take responsibilities, ability to keep up to date in many fields, ability to command the respect of others, a knack for utilizing the talents of others, and appreciation of people. Many of the attributes of a leader are qualities which are desired in a medical technologist.

These same attributes are frequently found among many different groups of scientists. In fact, where scientists have accepted positions of leadership, others have made such comments as, "She doesn't let anything worry her," or "She acts like there is nothing to it."

Scientists have learned to discipline themselves. They don't expect to learn by osmosis, but have learned to dig hard to learn the essential facts. They also go into positions which tax their intellects. They must apply the scientific principles learned in college, learn to reason, adapt the work of others, and put forth some physical labor. They have few opportunities to exercise showmanship, yet they actually produce at a high level, and use advanced knowledge frequently.

The mental training which a scientist gets helps to keep his mind alert, and when he is faced with leadership responsibilities he can take them on without much difficulty.

During the years when most medical technologists were on call frequently, the medical technologists were seldom available for civic duties such as making collections for the Community Chest, serving as an officer in an organization, or helping in a community center.

Medical technologists now have as much time as office workers, and more free time during the school year than teachers have. In the average city there are not more than two scientific meetings to be attended during a month, and many technologists attend school at night or otherwise make use of their free time.

Medical technologists frequently become active in Business and Professional Women's Clubs and American Association of University Women, and other members are almost invariably proud of the efficiency and intelligence of their scientific members.

Sometimes a person shows even more leadership in connection with activities not associated with her membership in some organization. The scientist is often a popular speaker for many types of groups in the schools and out. Chemistry clubs, Biology groups, Future nurses' organizations, and other school clubs are anxious to have speakers who know something about science. Advisors are sometimes chosen from outside the faculty. Sometimes a medical technologist is called upon to help in a science fair project. If a medical technologist is friendly with high school age young people they will ask for advice both in their proj-

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ects and their personal lives. They will visit the laboratory, use reference books to get information for their projects, and startle you by the great amount which they learn.

Non-classroom teaching is one of the pleasures of any scientist. Visitors old and young find life much richer after they are inspired by a medical technologist to find out more about the world of science.

One medical technologist always attempted to answer questions asked by casual vistors. One middle-aged widower stopped by the laboratory frequently, always asking one or two questions. The medical technologist showed him whatever she was looking at under the microscope. Finally he confessed that he had always wanted to be a doctor, so she suggested, "Why don't you?"

He couldn't give a good reason, so he finished college, went to medical school, and returned to that hospital as an intern.

Influencing adults to continue education is almost as important as guiding young students. The best antidote against mental maladjustment and worry is stimulating education obtained either in a school or from books and periodicals. Some people have lost interest to such an extent that they even neglect the daily newspapers.

Scientists are naturally alert people, and few medical technologists need to be encouraged to keep up to date in science and world news. They seldom exert their influence to keep other people alert. The medical technologist almost invariably knows what is going on, but she shyly listens while others carry on a conversation. Although silence is golden, the medical technologist owes it to her profession to let her associates know how alert and well informed she is. She should learn how to intersperse a few well chosen words between the periods of silence.

It takes time to acquire a reputation as a leader, good organizer, or outstanding citizen. The first requirement is to show your dependability. The person who always does his best frequently is called upon for jobs which must be done well. Some of these are thankless jobs, but many of them are difficult chores which bring considerable prestige to the person who does a good job.

In a small community the medical technologist is somewhat of a curiosity, but she is seldom called upon to speak of her profession until she has proved herself capable of performing some civic task. One medical technologist even became a volunteer in the hospital where she worked in order to show the women of the town that she wanted to be one of them. Within a year she was being called upon to talk to groups to explain the Red Cross Blood Bank. Later, she became a popular career speaker, and everyone from the cub scouts to the golden age club wanted to hear about medical technology. She had become an effective leader.

In larger cities the president of the ASMT or recruiting chairman is the first person contacted, and if she is a real leader she will have many others giving recruitment talks.

A good leader never allows herself to be overworked. She learns to delegate responsibility, and to give others a feeling of importance.

The medical technologist who is a leader becomes a source of information. In many communities there was little interest in civil defense preparations until medical technologists in a matter-of-fact way explained

their part in civil defense. He who wants to be a leader needs to train in order to carry out his duties effectively. The willingness to take training is a mark of leadership, and some technologists have held office in civil defense organizations. The orderly way in which they handle the duties leads to other invitations for civic services.

There are some people who get the idea that the leader is the person who gets up before a group to conduct meetings, but leadership is much more. A few people who don't like to speak before groups are still especially effective leaders.

It does pay, however, to learn to express oneself before a group. A leader who is willing to speak in public or over the radio is a much more valuable leader. The one who is willing to serve as a teacher, song leader, dance instructor, or discussion group leader is even more valuable.

In some communities there are few opportunities to show leadership. Many students feel a sense of frustration because they find no need for their leadership abilities outside of their jobs.

Libraries, recreation departments, great books discussion groups, and other organizations can give suggestions. One can frequently volunteer to help in some organization like the Tuberculosis Association or the Good Sports who do favors and provide transportation for the Indoor Sports.

One who sees something which needs to be done should never be shy about offering to do it whether it be washing dishes at a big dinner or tying a child's shoe lace. Occasionally a person is ridiculed for butting in, but more often he is admired for his willingness to work, and his talent for seeing what needs to be done.

Some people feel that the person who wants to be a leader should do whatever he is asked to do. The wise individual, however, never attempts to do what he is certain could be done by another person who would appreciate being asked. One medical technologist got a reputation for leadership because she always knew who would do a job well. She had a talent for getting others to talk about their dreams and aspirations. She then suggested that a person be given a chance to do the things which she had desired to do.

It is often surprising what good judges most people are of their own abilities. However, it takes a real leader to recognize that it pays to let people attempt to do those things which appear difficult and for which they have little preparation.

Medical technologists are usually natural leaders. They have developed the habit of thinking things through. They are not influenced by snap remarks nor swayed by a dramatic argument. A scientist on a committee often acts as a balance and thus prevents the group from making half-baked decisions.

Scientists are frequently sought for positions as officers because of the splendid way in which they perform, but even the scientist who does not hold any office in any organization is a leader. The scientists represent progress in a community, and the entire neighborhood looks to them for guidance. In a hospital medical technologists represent added efficiency; they mean that something new has been added. It is therefore important for medical technologists to be admirable people,

the kinds of persons who make young people want to be like them. Often children study much harder because they desire to measure up to another person, and medical technologists are wonderful persons to measure up to.

You are leaders whatever you are doing, the same type of leaders as many doctors. In some communities you can exert even more influence for effective education than the doctors wield because your accomplishments seem to be more within the reach of many young people. Whether your leadership is silent or vocal, many people are looking to you as a leader. They desire to know as much as you know, do the things you do, and therefore they will strive to learn as you have learned. If you add to that influence the help you can give by assisting in work or taking responsibility, you will be a valuable asset to your community, and you will exert a definite force toward better education for all people regardless of age.

Your greatest opportunity to help to make a better tomorrow comes when you have an opportunity to guide young people in the choice of a career. If you make science attractive, the best minds of the future will be attracted towards medical technology and related sciences. The greater the competition in school, the harder people will study, and the more they will learn.

As the requirements for medical technologists are raised, it will be necessary to increase recruitment efforts, and nothing will attract young people to a career as effectively as a charming, intelligent, enthusiastic leader whose manner says, "Wouldn't you like to be in my profession?"

Becoming a leader who is available for civic activity brings no immediate rewards, but the job satisfaction will help you become the type of person who in many ways makes the community better. And as you become a recognized leader, material returns will probably follow. But even if they do not, you will be a person who is making the world a better-informed place, and eventually it will be shown that Knowledge is Power, and you will be able to extend a guiding hand in leading others so that knowledge will be used to benefit the greatest number of people.